UNCLASS

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

(1)

REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER: AFIT/CI/NR 85-116T	2. GOVT ACCESSION NO: AD-A160 740
3. RECIPIENT'S CATALOG NUMBER:	
4. TITLE (and Subtitle): Glucocorticoid Antagonism by Endotoxin: Biological Effects During Stress and Basis for Inhibition of Phosphoenolpyruvate Carboxykinase	5. TYPE OF REPORT & PERIOD COVERED: THESIS/DISSERTATION
	6. PERFORMING ORG. REPORT NUMBER:
7. AUTHOR(s): William B. Huff	8. CONTRACT OR GRANT NUMBER(s):
9. PERFORMING ORGANIZATION NAME AND ADDRESS: AFIT STUDENT AT: The University of Texas Austin	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS:
11. CONTROLLING OFFICE NAME AND ADDRESS: AFIT/NR WPAFB OH 45433 - 6583	12. REPORT DATE: August 1985
	13. NUMBER OF PAGES: 129
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office):	15. SECURITY CLASS. (of this report): UNCLASS
	15a. DECLASSIFICATION/DOWNGRADING SCHEDULE:

16. DISTRIBUTION STATEMENT (of this Report)

APPROVED FOR PUBLIC RELEASE; DISTRIBUTION UNLIMITED

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

APPROVED FOR PUBLIC RELEASE: IAW AFR 190-1

LYNN E. WOLAVER
Dean for Research and
18 Oct 85 Professional Develop
AFIT, Wright-Patterson

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

ATTACHED

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE UNCLASS

SECURITY CLASSIFICATION OF THIS PAGE (When Data

REPRODUCTION QUALITY NOTICE

This document is the best quality available. The copy furnished to DTIC contained pages that may have the following quality problems:

- Pages smaller or larger than normal.
- Pages with background color or light colored printing.
- Pages with small type or poor printing; and or
- Pages with continuous tone material or color photographs.

Due to various output media available these conditions may or may not cause poor legibility in the microfiche or hardcopy output you receive.

☐ If this block is checked, the copy furnished to DTIC contained pages with color printing, that when reproduced in Black and White, may change detail of the original copy.

AFIT RESEARCH ASSESSMENT

The purpose of this questionnaire is to ascertain the value and/or contribution of research accomplished by students or faculty of the Air Force Institute of Technology (AU). It would be greatly appreciated if you would complete the following questionnaire and return it to:

AFIT/NR
Wright-Patterson AFB OH 45433

RESEARCH TITLE: Glucocorticoid Antagonism by Endotoxin: Biological Effects During Stress and Basis for Inhibition of Phosphoenolpyruvate Carboxykinase

AUTHOR: William B. Huff

RESEARCH ASSESSMENT QUESTIONS:

1. Did this research contribute to a current Air Force project?

( ) a. YES ( ) b. NO

2. Do you believe this research topic is significant enough that it would have been researched (or contracted) by your organization or another agency if AFIT had not?

( ) a. YES ( ) b. NO

3. The benefits of AFIT research can often be expressed by the equivalent value that your agency achieved/received by virtue of AFIT performing the research. Can you estimate what this research would have cost if it had been accomplished under contract or if it had been done in-house in terms of manpower and/or dollars?

( ) a. MAN-YEARS _____ ( ) b. $_____

4. Often it is not possible to attach equivalent dollar values to research, although the results of the research may, in fact, be important. Whether or not you were able to establish an equivalent value for this research (3. above), what is your estimate of its significance?

( ) a. HIGHLY SIGNIFICANT ( ) b. SIGNIFICANT ( ) c. SLIGHTLY SIGNIFICANT ( ) d. OF NO SIGNIFICANCE

5. AFIT welcomes any further comments you may have on the above questions, or any additional details concerning the current application, future potential, or other value of this research. Please use the bottom part of this questionnaire for your statement(s).

NAME GRADE POSITION

ORGANIZATION LOCATION

STATEMENT(s):

GLUCOCORTICOID ANTAGONISM BY ENDOTOXIN: BIOLOGICAL EFFECTS DURING STRESS AND BASIS FOR INHIBITION OF PHOSPHOENOLPYRUVATE CARBOXYKINASE

by

William B. Huff, Capt, USAF, BSC

THESIS

129 Pages

Presented tc the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

MASTER OF ARTS

THE UNIVERSITY OF TEXAS AT AUSTIN

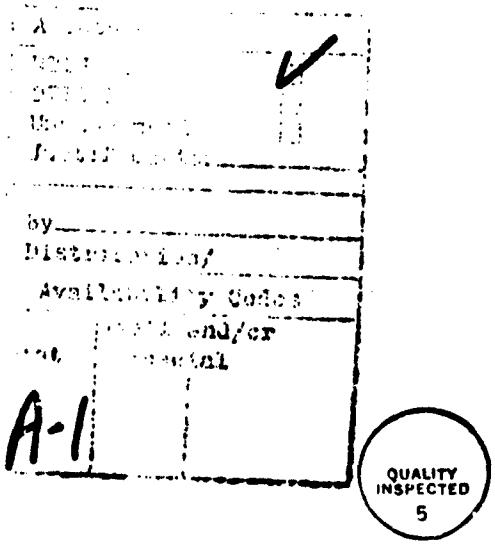
August, 1985

GLUCOCORTICOID ANTAGONISM BY ENDOTOXIN: BIOLOGICAL EFFECTS DURING STRESS AND BASIS FOR INHIBITION OF PHOSPHOENOLPYRUVATE CARBOXYKINASE

APPROVED:

L. Lee Berry

Jan J. Mohrenweiser



GLUCOCORTICOID ANTAGONISM BY ENDOTOXIN: BIOLOGICAL EFFECTS DURING STRESS AND BASIS FOR INHIBITION OF PHOSPHOENOLPYRUVATE CARBOXYKINASE

by

William B. Huff, B.S.

THESIS

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

MASTER OF ARTS

THE UNIVERSITY OF TEXAS AT AUSTIN

August, 1985

ACKNOWLEDGEMENTS

This author wishes to extend a special gratitude to Dr. L. J. Berry for his encouragement and support during my tenure in his laboratory. In addition, acknowledgement is given to my wife Susan and my two sons Joshua and Justin for their patience with, and understanding of, the many hours necessary for compilation of this thesis.

ABSTRACT

Bacterial endotoxins produce a variety of pathophysiologic effects by inducing the release of humoral factors from cells of the reticuloendothelial system, primarily macrophages. This study focusses on one particular factor, glucocorticoid antagonizing factor (GAF). Antagonism was augmented when mice were subjected to conditions of stress where adrenoglucocorticoids are necessary for survival. Mice were sensitized to both toxic endotoxin and irradiated detoxified endotoxin during the stress of cold, heat, and tourniquet shock. Endotoxin, as well as detoxified endotoxin, induced the release of GAF from cells of the RES. Treatment of mice with this GAF-rich serum also resulted in their sensitization to stress.

Survival of LPS-poisoned animals subjected to stress could be improved when exogenous hydrocortisone was given prior to the administration of exogenous GAF-rich serum or prior to the release of endogenous GAF in response to endotoxin. Naloxone, a B-endorphin antagonist, failed to increase the survival rate of animals under the experimental conditions investigated.

Endotoxin given 1 hour prior to hydrocortisone or GAF-rich serum given concurrent with the hormone inhibited the induction of the gluconeogenic rate-limiting enzyme phosphoenolpyruvate carboxykinase (PEPCK). It was shown that

uninducible catalytic activity was not a result of suppressed or modified catalysis as the amount of enzyme, measured by ELISA, correlated directly with catalytic activity as measured by carboxylation with radiolabeled bicarbonate.

The data reported in this thesis suggest that GAF produces a pharmacological antagonism of the animals ability to manifest an adrenal cortical response. This antagonism may be the determining factor in the animals demise during conditions of stress and it is characterized by impairment of carbohydrate homeostasis due to noninduction of PEPCK.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figures: Page:

INTRODUCTION

Almost a century ago Boise noted that infection preceded a state of septic shock (26). Another half-century passed before the hemodynamic alterations associated with gram-negative septic shock were directly attributed to a cell wall constituent referred to as endotoxin or lipopolysaccharide (LPS) (103,166). Since that time bacterial LPS has been under extensive investigation. Although much has been learned about the pathophysiology of this toxin, today it still stimulates the curiosity of many researchers due to the undefined manner by which LPS precipitates death during gram-negative sepsis and the associated endotoxin (septic) shock.

Septic shock can be induced by a wide variety of microorganisms. These include gram-negative bacteria (56, 163), gram-positive bacteria (4), spirochetes (87), rickettsia (91), and fungi (32). Endotoxin shock caused by gram-negative bacteria is of special interest because of its continued prominance despite antimicrobial therapy (122,172). Sepsis associated with gram-positive bacteria is complicated by the clinical incidence of shock in less than 5 percent of the cases (143,144). This is contrasted to gram-negative bacterial sepsis where approximately 30 percent of the bacteremic patients develop septic shock and the average mortality rate of these patients is greater than 50 percent. This is compared to a mortality rate of less than 20 percent

when gram-negative sepsis is uncomplicated by shock (70,105, 122,168). Thus, the importance of resolving the nature of how LPS exerts its metabolic effects is clearly indicated.

The events of endotoxin shock can be divided into early and late phases based upon the pathophysiologic effects on the circulatory system (17,122). Immediate hemodynamic alterations include an initial vasoconstriction followed by vasodilation with an initial increase followed by a decrease in systemic arterial blood pressure (79,167). Leukocytosis is followed by leukopenia (153) and a hypercoagulable state precedes a hypocoagulable state due to disseminated intravascular coagulation (DIC) with depletion of clotting factors (45,46). These early events lead to arteriovenous shunting, microvascular insufficiency and anoxia due to inadequate perfusion of tissues (122). Ultimately, progression to irreversible shock characterized by terminal hypotension, severe organ dysfunction and severe myocardial depression are believed to contribute to the animals demise (17, 62,122).

The exact nature of an initial mechanism(s) leading to irreversible shock and death has not been defined and several mediator substances released by endotoxin-mediated shock may be involved. No intention is made to slight any authors contributions in this important field of investigation but time constraints will limit this discussion to only a brief overview of those mediators which have been implicated as initiators of irreversible shock. Endotoxinemia

elicits the release of endogenous histamine from intravascular as well as extravascular storage sites (76,77). In addition, elevated histidine decarboxylase activity occurs during challenge with LPS or stress (141). In an effort to correlate histamine production with endotoxin shock it was shown that exogenous histamine, LPS, or histamine releasor agent, known as 48/80, produce increased portal venous pressure with subsequent pooling of blood in the hepatosplanchnic area, decreased venous return, and decreased systemic arterial pressure in dogs (78). These data implicate histamine as a causitive factor of hypotension seen during endotoxin shock. Further support for similar pathophysiologic effects of histamine and LPS was shown when phenoxybenzamine, an anti-histaminic agent, prevented increased portal venous pressure and pooling of blood upon exposure of dogs to either LPS or histamine (31). However, decreased systemic arterial pressure was not blocked by phenoxybenzamine in LPS-poisoned dogs even though blockage did occur in dogs treated with histamine (31). It was also shown that phenoxybenzamine could not afford protection against systemic hypotension or death of endotoxin shocked dogs (31,106). In addition, others have reported that the LPS-induced acute increase in portal venous pressure and decrease in systemic arterial pressure observed in dogs was not an acute episode in cats, rabbits, or monkeys (79,93). Therefore, it appears that histamine does not play a major role in the initiation of irreversible

endotoxin shock and consequently other factor(s) are implicated.

A proposed model for the pathophysiology of endotoxin shock suggests that LPS interacts with antibody and complement. The subsequent generation of C3a and C5a in turn activates leukocyte aggregation and adherence to vascular endothelium where the release of lysosomal enzymes and very potent toxins, superoxide and hydrogen peroxide occurs. These can cause endothelial damage leading to endogenous volume loss and hypotension (88,97). The basis for this model is an initial reaction between LPS and anti-LPS antibody. This is quite reasonable since most mammals contain antibodies to LPS (63,95). It is well known that LPS activates the complement cascade (42,68,152). Fearon et al (55) showed that complement activation correlates with LPS-induced hypotension and death. Furthermore, increased vascular permeability can be aborted by protecting the vascular endothelium from the insult of complement activation (156). This was shown to be true in neutropenic animals, ie, the lack of response that occurs as a result of the elimination of C5a responder and of oxygen radical producing cells and by the protection afforded these animals by treatment with superoxide dismutase (156). Also, dogs undergoing exchange transfusion with plasma that had been made hypocomplementary by heating at 56°C were refractory to the lethal effects of LPS (151). However, others have shown that during hypocomplementemia

that was induced by treatment with cobra-venom factor (CVF) or by congenital C6 deficiency, the initial (ie, within the first few minutes after challenge with LPS) hypotension is not manifested but that overall, hypotension and mortality is unaltered (60,161). Therefore, complement may be involved in an initial LPS-induced hypotension, but the terminal hypotension must be explained by another mechanism.

Catecholamine production has also been shown to occur during endotoxinemia (25,135). It is believed that in an effort to maintain homeostasis, elevated histamine levels cause the release of these adrenergic amines from the adrenals (31,35,78). This concept is supported by showing hypersensitivity of rats and mice to histamine following adrenalectomy (71). This hypersensitivity could be counteracted by administering exogenous epinephrine and it thus appears that catechols and amines exert opposite and compensatory effects on one another (71,150). In addition, increased urinary excretion of epinephrine and norepinephrine was observed in man following subcutaneous injections of histamine (51). However, catecholamine production during endotoxin shock does not appear to be a major factor in the irreversible phase of shock as adrenalectomized animals exhibited no significant change in portal hypertension or systemic hypotension relative to intact animals when given LPS (30,80). Also catecholamine antagonists, such as reserpine, do not abolish LPS-induced hypotension (89). These results indicate

that catecholamines are not responsible for irreversible endotoxin shock.

Endogenous opiates (endorphins) have drawn recent interest as mediators of endotoxinemic shock. Adrenocorticotropin hormone (ACTH) and B-endorphin , the most potent endorphin, are stored in a common pituitary site (69,137) and are synthesized from the same precursor glycoprotein (69,104). Both ACTH and B-endorphin are elevated in a number of stressed states, including LPS-poisoning (69,81,110,137). Exogenous endorphins have been shown to produce significant hypotension in rats and dogs (81,98). Therefore, these endogennus opiates may play a critical role in the classical LPS-induced hypotension. This is substantiated by studies with naloxone, a B-endorphin antagonist, which can reverse the hypotension seen with LPS challenge (52,53,81,98). This is a selective reversal of pathophysiologic effects and not a direct pressor mechanism as naloxone given to nonendotoxinemic rats or dogs does not alter systemic blood pressure (52,81). Although naloxone reverses hypotension in endotoxinemic rats it has no effect on their survival rate (53). Thus, in the rat system at least, there appear to be factors other than hypotension and cardiac collapse leading to death. This is different from LPS-poisoned dogs, where naloxone not only attenuates hypotension but also increases the survival rate (52). This work has also been extended to primates: in Cynomolgus monkeys naloxone has been shown to improve

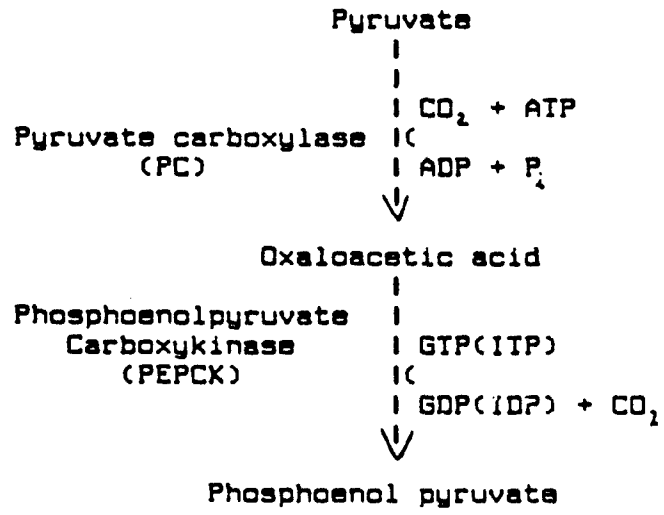
hypotension and survival following endotoxinemia (83). In addition to reversing hypotension, naloxone also prevents other common features of septic shock, eg, the initial hyperglycemia in mice due to depletion of hepatic glycogen stores (73). The depletion of hepatic glycogen and other carbohydrate anomalies may be critical in the demise of LPS-poisoned animals as hypoglycemia is a hallmark of terminal endotoxin shock (10,58,94).

Total depletion of hepatic glycogen during LPS-challenge results in an initial, but transient hyperglycemia, followed by a profound and terminal hypoglycemic state (19,58,94). Adrenalectomy, with subsequent corticosteroid replenishment, abolished this LPS-induced glycogen depletion (139,153). Therefore, enhanced glycogenolysis during endotoxinemia is likely due to the release of catecholamines from the adrenal glands. Elevated glycogenolysis by itself however, should not account for terminal hypoglycemia. An increase in carbohydrate consumption and/or hepatic gluconeogenesis should compensate for any glycogenolytic derangement. Unfortunately, both of these parameters are blocked by LPS (58,94,159).

Using a phenol red solution as a tracer, Turner and Berry (159) showed that gastric emptying is prevented within 5-10 minutes after exposure to LPS. Prior to this, it had been shown that LPS suppresses gastric motility (117). Since gastric contractions assist in the elimination of chyme from

the stomach it appears that the inhibition of gastric emptying is due to decreased gastric motility. the overall effect is essentially a condition of starvation due to decreased food and water uptake during endotoxinemia. Since LPS blocks the consumption of exogenous carbohydrates the animal becomes dependent on hepatic gluconeogenesis to neutralize terminal hypoglycemia.

Gluconeogenesis, or reverse glycolysis, depends on the reversal of three highly exergonic reactions (146): glucose-6-phosphate to glucose, fructose-1,6-diphosphate to fructose-6-phosphate, and pyruvate to phosphoenolpyruvate. The former two reactions are catalyzed by hydrolysis of ester phosphate bonds by the enzymes glucose-6-phosphatase and fructose-1,6-diphosphatase, respectively, while the latter reaction involves the coupling of two nucleoside triphosphate cleavage reactions, as shown below.



Endotoxin inhibits the induction, by glucocorticoids or fasting, of each of these enzymes: glucose-6-phosphatase (101), fructose-1,6-diphosphatase (101), and PEPCK (16). Although it may be unusual for a single enzyme to control the expression of an entire metabolic pathway, there is strong evidence to suggest that PEPCK is the rate-limiting enzyme in gluconeogenesis; therefore, the interaction of LPS with PEPCK will be emphasized. Seubert et al (146) measured gluconeogenesis by the formation of glucose in hepatic or renal cortex slices from animals receiving cortisol 6 hours ealier. The amount of glucose formed was directly proportional to the activity levels of pyruvate carboxylase and PEPCK, but not to glucose-6-phosphatase or fructose-1,6-diphosphatase. They concluded that the conversion of pyruvate to phosphoenolpyruvate is the rate-limiting step in gluconeogenesis. Rongstad (134) showed PEPCK to be the rate-limiting enzyme by using 3-mercaptopicolinate, a strong inhibitor of PEPCK. Inhibition of PEPCK correlated directly with the reciprocal rate of synthesis of glucose in rat hepatocytes. Since PEPCK appears to be the regulatory enzyme in gluconeogenesis, further details of its role and regulation are warranted.

PEPCK was first observed in chicken liver mitochondria in 1954 (162). It is found primarily in the cytosol of hamsters, rats, and mice and in the mitochondria of rabbits and chickens (72), and equally proportioned in the cytosol

and mitochondria in man (155). Ballard and Hanson showed the cytosolic and mitochondrial isoenzymes to be immunologically distinct proteins. While some investigators have shown that only the cytosolic form responds to diet and hormones (118,148), others have implicated a role for mitochondrial PEPCK (3,72). Although details of the physiologic interaction between cytosolic and mitochondrial PEPCK has yet to be resolved, they both appear to be regulators of gluconeogenesis (155).

Hepatic PEPCK is readily induced by glucagon (a cAMP stimulator) (127,169), cAMP (127,169,170), norepinephrine (127), glucocorticoids (118, 126,148,170), fasting (59,148), alloxan or mannoheptulose induced diabetes (148), and thyroxin (28,116). As a negative control, insulin inhibits the induction of hepatic PEPCK (67,149,169,170). This appears to be a direct suppression as insulin, in the absence of glucose (a glucagon, hence, cAMP modulator), inhibits the dibutyryl cAMP induction of PEPCK activity in Reuber H-35 cells (67,154). It should be noted that adrenalectomy or induced diabetes does not interfere with the circadian cycle of PEPCK (123). Thus, multiregulation of this enzyme is apparent and when one regulatory mechanism becomes compromised the other(s) may compensate.

Recent attention has been given to investigating the level at which hepatic PEPCK is regulated. Since actinomycin D blocks the induction of PEPCK by cAMP (169) or glucocorti-

coids (130,131), regulation at the level of transcription is probable. This is substantiated by measuring the increase in amount of immunoprecipitable PEPCK from in vitro translation of hepatic RNA following treatment with cAMP (84,85) or glucocorticoids (85,107). In addition, a direct measure of glucocorticoid or cAMP induced levels of mRNA for PEPCK using cDNA probes has also confirmed a transcriptional level of regulation (2,8,38,39,164). With this background of PEPCK and its regulation, a discussion of how LPS interacts with this crucial gluconeogenic enzyme will ensue.

Kun (94) was the first to observe an inhibition of gluconeogenesis by LPS when he noted severe hypoglycemia in LPS-poisoned rats. Other studies have confirmed this early report. Shands et al (147) showed that LPS neither altered the rate of glucose clearance from the blood nor altered oxygen consumption, measured by metabolism and oxidation of various substrates. They concluded that LPS-induced hypoglycemia was due to inhibition of gluconeogenesis and not due to insulin or increased metabolism. McCallum and Berry (102) saw an almost 50 percent inhibition of incorportaion of [U-^{14}C]alanine into blood glucose after LPS-poisoning in mice. Additional studies have also shown impaired gluconeogenesis upon endotoxinemia (58,113,171). The question remained however, as to how LPS inhibits gluconeogenesis. Rippe and Berry (18,130,131) reported the inhibition of induction of hepatic PEPCK following either LPS-poisoning or

actinomycin D. Although LPS inhibits the induction of other hepatic enzymes, glucose-6-phosphatase (101), fructose-1,6-diphosphatase (101), and tryptophan oxygenase (16,132), this is not due to gross hepatic tissue damage since it does not inhibit the induction of hepatic tryosine amino transaminase (16). The above data clearly show that LPS inhibits gluconeogenesis at the level of PEPCK. Several studies have provided insight as to how this antagonism occurs.

Using a compilation of reports, Berry (17) suggested LPS antagonizes the glucocorticoid induction of PEPCK by a a humoral factor. First, the large size of endotoxin made it an unlikely intracellular toxin (121). After endotoxinemia, Noyes (121) detected LPS in phagocytic Kuppfer cells in the liver but not in nonphagocytic hepatic parenchymal cells. Second, aimals with a hyperactive reticulcendothelial system (RES) are sensitized to the lethal effects of LPS (9,44). Thus, the basis for an RES mediating substance was noted and subsequent studies supported this.

Moore et al (111) showed serum from LPS-poisoned mice inhibited the glucocorticoid induction of PEPCK in mice made tolerant to LPS. Tolerant mice are refractory to the effects of LPS. In addition, supernatant from peritoneal exudate cells (PEC's), primarily macrophages, also inhibited the glucocorticoid induction of PEPCK in mice made tolerant to LPS (111). Furthermore, normal mice pretreated with rabbit anti-macrophge serum did not exhibit LPS inhibited PEPCK

activity (111). Pretreatment with anti-thymocyte serum did not abolish the inhibition of PEPCK activity by LPS (111). This investigation clearly establishes an LPS-induced humoral substance produced by macrophages of the RES which antagonizes the glucocorticoid induction of PEPCK and was thus called 'glucocorticoid antagonizing factor (GAF)' (111). Other studies have since confirmed GAF as the LPS-induced mediator which inhibits the glucocorticoid induction of hepatic PEPCK.

Congenitally athymic nude mice (nu/nu) exhibit hydrocortisone induced PEPCK activity regardless of LPS treatment (112). Their PEPCK activity is, however, inhibited by serum rich in GAF (ie, serum collected 2 hours after administration of LPS to mice pretreated with zymosan, an RES activator) (112). In addition, C3H/HeJ mice, which do not respond to LPS and exhibit a fully inducible PEPCK activity even in the presence of LPS, resist hydrocortisone induced enzyme levels in the presence of GAF-rich serum (114). Also, Reuber H-35 hepatoma cells which are refractive to the LPS inhibition of hydrocortisone induced PEPCK activity, displayed total inhibition of PEPCK activity when treated with GAF-rich serum (65,66). Finally, Couch et al (43) showed that mice made tolerant to LPS and subsequently placed in the cold were sensitized to the lethal effects of GAF-rich serum but not LPS.

It is the purpose of this paper to evaluate the

glucocorticoid antagonism of LPS, to further support the role of GAF as an LPS-induced mediator, and to assess the dysfunction of carbohydrate metabolism at the level of PEPCK by either LPS or GAF.

MATERIALS AND METHODS

CHEMICALS:

All chemicals, reagents, and enzymes used in these experiments were obtained from Sigma, unless otherwise noted.

ENDOTOXIN (LPS):

Commercially prepared and lyophilized LPS from Salmonella typhimurium was purchased from Difco, Detroit, MI, cat # 3125-25. It was stored dessicated at 4 C and reconstituted in sterile, nonpyrogenic 0.9% isotonic saline. The reconstituted solution was stored at -20 C. Freeze thawing did not alter, in a detectable way, the activity of LPS.

RADIATION DETOXIFIED ENDOTOXIN (RD-LPS):

This preparation was graciously provided by Dr. Lorand Bértók of the National Institute of Radiobiology and Radiohygiene in Budapest, Hungary. It is a phenol-water endotoxin preparation from Escherichia coli 089, human strain, which was detoxified by exposure to 150 kGy (kiloGrays) of 60-Co gamma irradiation. Reconstitution and storage was the same as that for toxic LPS.

ANIMALS:

CFW mice initially purchased from Charles River breeding Laboratories Inc. (Wilmington, MA) and subsequently

bred at this facility were employed in all experiments. Both male and female mice were used, ranging in weight from 20 to 25 grams. Mice were housed no more than 12 per large cage or 5 per small cage with Beta Chip bedding (Northeastern Products Corp., Warrenburg, N.Y.). Food pellets from Wayne Lab-Blox, Allied Mills, Chicago, Il. and tap water were available at all times except during controlled starvation.

TOLERANCE:

Mice were made tolerant to LPS by a modification of the procedure described by Goodrum and Berry (66). A series of intraperitoneal injections were given at increasing dose-levels of 10 ug, 15 ug, and 25 ug LPS in volumes of 100, 150 and 250 ul prepared from a 100 ug/ml solution. Mice were injected on days 1,2 and 4, respectively, prior to challenge 48 hours after the last injection. Mice rendered tolerant do not respond to the lethal effects of LPS.

STIMULATION OF THE RETICULOENDOTHELIAL SYSTEM (RES):

Hyperactivation of the RES sensitizes mice to LPS and it also increases the yield of induced humoral factors known to be produced by macrophages. In order to stimulate the RES, mice were given an intravenous injection of 700 ug of heat-killed Corynebacterium parvum (Burroughs Wellcome, cat. # CN 6134) in a total volume of 100 ul. Mice were then challenged 6 days later.

COLLECTION OF GAF-RICH SERUM (CpES):

GAF (glucocorticoid antagonizing factor) rich serum, also called Corynebacterium parvum - endotoxin serum (CpES), was collected by decapitating mice 2 hours after an intravenous injection of 25 ug LPS in mice whose RES had been stimulated with C. parvum 6 days earlier. Blood was allowed to clot approximately 3 hours at 4°C then centrifuged at 10,000 rpm for 10 minutes. The serum was filtered through a 0.45 um millipore filter and stored at -20°C.

COLD AND HEAT STRESS MODELS:

Cold stress was induced according to Couch and Berry (43). Endotoxin tolerant mice were placed individually, without food, water, or bedding on a wire mesh screen, about 1.5 cm above the table top in a walk-in refrigerator at 5°C. Each mouse was covered with a 250 ml beaker. A brick, or other weight, was placed on top of the beaker to prevent the mouse from escaping. Temperature was regulated between 4°C and 5°C. Heat stress was induced by housing endotoxin tolerant mice in a 37°C walk-in incubator. There were 5 mice per cage and bedding, but no food or water was provided. No method for controlling humidity in either the cold or heat stress environment was available. Rectal temperatures were measured with a small animal thermistor, inserted about 1 cm into the rectum and read on a telethermometer (Yellow Springs Instrument Co.) after 3 minutes. Temperatures were

recorded every hour.

TOURNIQUET SHOCK STRESS MODEL:

Tourniquet shock was induced by a modified version of that of Rosenthal (136). To a 1500 ml glass beaker was added about 50 ml chloroform. A wire mesh stand was placed into the beaker and the top of the stand was approximately 10 cm above the liquid chloroform surface. A single mouse was placed onto the wire mesh and aluminum foil was then placed over the beaker. Mice were anesthetized for 15-30 seconds. It is better to re-anesthetize than to over-anesthetize. The subdued mouse was then placed in a hollow brass cylinder about 4 cm in diameter. A rubber band (size 30, measuring 2 x 1/32 x 1/8 in) was then wrapped in 6 equal turns around a tuberculin syringe case, 1 cm in diameter and 3 cm long. This hollow case was coated lightly with glycerol for easy removal of the rubber band. To the hind leg of the mouse was tied, by a slip knot, fishing line attached to an 11 gm lead weight. The weight was passed through the TB syringe case pulling the hind leg into the case. The rubber band was then slipped off the case so that it fit snugly against the body and around the proximal hind leg. The fishing line was then removed and the process was repeated for the other leg. Tourniquets were left on for 1 hour before removal with sharp scissors.

PHOSPHOPOENOLPYRUVATE CARBOXYKINASE PURIFICATION:

This procedure is a modification of that of Colombo (41) and Iynedjian (86). Induced levels of PEPCK were obtained by starving mice 24 hours prior to challenge. After this time, mice were decapitated, their livers were perfused with ice cold sterile, nonpyrogenic 0.9% isotonic saline via the portal vein. The gall bladder was excised and discarded. The livers were excised and immediately frozen in liquid nitrogen. Livers were maintained at -20°C for a maximum of 48 hours until purification was begun. All purification precedures were performed at 4°C. Each liver was homogenized in 5 volumes (w/v) of buffer 1 (50 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, 0.25 M sucrose, pH 7.4) by a motor driven teflon Potter-Elvehjam homogenizer at one-quarter speed. The homogenate was then centrifuged at 100,000 x g for 1 hour at 4°C in a Beckman Ti50 rotor using a Beckman model L ultracentrifuge. Solid ammonium sulfate, 45% (w/v), was added to the supernatant and stirred overnight at 4°C and the precipitate was removed by centrifugation at 10,000 x g for 15 minutes. The supernatant was then made 65% (w/v) in ammonium sulfate. This was stirred for 4 hours at 4°C. The precipitate was collected after centrifugation at 10,000 x g for 15 minutes and dissolved in buffer 2 (50 mM Tris-HCl, 0.5 mM EDTA, 0.5 mM DTT, pH 7.5). It was desalted by overnight dialysis against buffer 2. Further desalting was accomplished by passing it through a 2.3 cm x 26 cm BioGel

P-6 column equilibrated with buffer 2. Eluate fractions with OD 280 greater than 0.15 were pooled and applied to a 2.7 x 9 cm DEAE-cellulose column equilibrated with buffer 2. This column was washed with buffer 2 until the OD 280 was zero and the PEPCK fraction was then eluted with a 300 ml gradient of 0 to 300 mM NaCl in buffer 2. The PEPCK fraction eluted between 70 and 150 mM NaCl. Fractions containing PEPCK activity, assayed by the method of Colombo (41), were pooled and concentrated to 5 ml by pressure filtration using a 43 mm Amicon PM 30 membrane at 15 psi nitrogen. This solution was diluted to 40 ml with buffer 3 (10 mM potassium phosphate, 0.5 mM EDTA, 0.5 mM DTT, pH 7.0) then dialyzed 24 hours against 2 changes of buffer 3. The dialysate was then applied to a 2.6 x 4 cm hydroxylapatite column equilibrated with buffer 3. This column was flushed with buffer 3 until the OD 280 read zero. The fraction containing PEPCK was then eluted with a 200 ml linear gradient of 0 to 200 mM potassium phosphate, pH 7.0. Fractions containing PEPCK activity were pooled and concentrated to 25-35 ml using the Amicon described above. This solution was then dialyzed for 2 days against 3 changes of buffer 4 (10mM tris-HCl, 0.1 mM EDTA, 0.1 mM DTT, 10% glycerol). The dialysate was then made 0.6 mM with manganese chloride and applied to a 1.2 x 2.4 cm agarose-hexane-GTP affinity column. this column was washed with 10 ml of buffer 4 (without manganese chloride). PEPCK was then eluted with buffer 4 containing 1 mM GTP in a

volume of 1-2 ml. Fractions containing PEPCK activity were stored at -20°C in a 50 percent glycerol solution.

SDS-PAGE:

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was by the method of Laemmli (95). Thoroughly cleaned glass plates were sealed on three sides using spacers and 1% hot agarose. These plates allowed the formation of a gel 1.5 mm in thickness. The separating gel was made in a 125 ml side-armed flask containing 10% acrylamide (Eastman), 0.26% bisacrylamide (Eastman), 0.375 M Tris-HCl, pH 8.8, 0.1% SDS, 0.05% N,N,N',N'-tetramethylethylenediamine (TEMED). Following degassing of the solution, polymerization was initiated with freshly made 10% ammonium persulfate (BioRad) to 0.015%. This solution was gently poured between the sealed plates and allowed to polymerize for 25 minutes at 25°C. To ensure a flat surface the solution was overlaid with a 1:4 dilution of lower buffer (1.5 M Tris, 0.4% SDS) during polymerization of the acrylamide. The stacking gel was prepared in essentially the same manner as the separating gel except the acrylamide and bisacrylamide concentrations were halved and the buffer was changed to 0.125 M Tris-Cl, pH 6.8. The overlay buffer was removed and after initiating polymerization with ammonium persulfate the stacking gel was placed on top of the sepparating gel. A teflon well-forming comb was inserted into the stacking gel

and polymerization occurred at 25°C in 20 minutes. The teflon comb was gently removed and the plates were placed in an electrophoretic apparatus containing electrode buffer (25 mM Tris, 0.192 M glycine, 0.1% SDS, pH 8.6). Samples were prepared by boiling for 5 minutes in an equal volume of sample buffer (62.5 mM Tris-Cl, pH 6.8, 4% SDS, 18% glycerol, 10% B-mercaptoethanol, 0.002% bomophenol blue). The samples were loaded (5-50 ul) using a Hamilton syringe into the sample well chambers. Molecular weight markers were run with each gel. Gels were electrophoresed for 4 hours at 25 mA, constant current, or until the bromophenol blue tracking dye was 1 cm from the bottom of the gel. Gels were fixed for 30-60 minutes in 10% acetic acid, 30% methanol, 10% trichloroacetic acid (TCA), stained for 30-60 minutes in 0.1% Coomassie brilliant blue R, 10% acetic acid, 30% methanol, and then destained overnight in 15% acetic acid.

ANTI-PEPCK IgG FRACTION ISOLATION:

On day 1, a New Zealand white rabbit was injected with a total volume of 2.0 ml containing 3 mg/ml purified PEPCK in an equal volume of complete Freunds adjuvant (Difco, Detroit, MI, cat. # 0638-600). This antigen-adjuvant mix was given as a 1.0 ml intraperitoneal and 0.25 ml subcutaneous injection in all four haunches. On days 24 and 26, 0.5 mg/ml purified PEPCK in a total volume of 1.0 ml sterile, nonpyrogenic 0.9% isotonic saline was injected intravenously. On

day 35, and twice a week thereafter for 4 weeks, the rabbit was bled by cardiac puncture using a 30 cc syringe and an 18 gauge needle. Approximately 15 ml of serum was collected from each bleed. The IgG fraction was isolated from this immune serum according to the method described by Nowotny (119). The serum was centrifuged at 10,000 x g for 15 minutes to remove any cellular debris. To this was added saturated ammonium sulfate dropwise to 40% saturation at 25°C while stirring. After 30 minutes the solution was centrifuged at 1000 x g for 30 minutes. The pellet was washed once with phosphate buffered saline (PBS, 0.02 M phosphate, 0.9% NaCl, pH 7.0) containing 40% ammonium sulfate and the washed pellet was redissolved in PBS to 10% of the starting volume. This solution was dialyzed every 8-12 hours against 2-3 buffer changes of 0.0175 M phosphate buffer (PB), pH 6.3, until no sulfate could be detected in the dialysis buffer when mixed with solid barium chloride. The dialyzed solution was clarified by centrifugation at 10,000 x g for 15 minutes and the clear supernatant was diluted to approximately 15 mg/ml with PB, pH 6.3. This solution was then applied to a 2.3 x 25 cm DEAE-Sephadex A-50 column equilibrated with PB, pH 6.3. The first fractions, which contain IgG, with an OD 280 were pooled and concentrated to approximately 3 mg/ml by pressure filtration using a PM 10 Amicon filter under 15 psi nitrogen. This solution concentrate was divided into 1.0 ml aliquotes and stored at -20°C.

TITRATING ANTI-PEPCK IgG:

Rabbit anti-mouse PEPCK IgG fraction was titrated by its ability to bind and precipitate out PEPCK from high-speed mouse liver supernatants. This procedure is a modified version of that of Ballard and Hanson (5). In 1.5 ml Eppendorf tubes, anti-PEPCK was diluted in 0.0175 M phosphate buffered saline, pH 6.3, to yield a total volume of 50 ul of various concentrations (0-250 ug) of the antibody. To this was added 10 ul of 25-30% (w/v) high-speed (100,000 x g) mouse liver supernatant. After mixing, the solution was incubated for 1 hour at 0°C. Preimmune IgG fractions were included as controls. Following the first incubation, 0.2 ml of a 10% suspension of heat-killed, formalin-fixed Staphylococcus aureus, Cowan I strain (Sigma cat. # P-7155, binding capacity of 1.56 mg human IgG per ml) was added and after gentle mixing was incubated for 15 minutes at 0°C. After centrifugation for 1 minute, 0.4 ml of the supernatant was assayed for PEPCK by the radiometric assay described later.

IMMUNODIFFUSION:

This was performed according to Clausen (40). A solution of 1% purified agar (Difco cat. # 0560-01) in veronal buffer (15 mM diethyl barbituric acid, 75 mM sodium barbital, pH 8.6) was prepared. Then 2.8-3.0 ml of molten agar was pipetted onto a glass slide cleansed with 70% ethanol. This was allowed to harden for about 5 minutes in

a moist humidifier. After punching an appropriate pattern with a Gelman punch, 3 mm bore, the wells were removed by vacuum. To the appropriate wells was added 10 ul of antigen (high-speed, 100,000 x g, mouse liver supernatant) and antibody (rabbit anti-mouse PEPCK). Diffusion occurred overnight and the immune precipitin line was read using a back-lighting apparatus (Kallestad Labs, Inc., Minneapolis, MN).

IMMUNOELECTROPHORESIS:

Modification of the procedure described by Nowotny (120) was used. 1% Purified agar, as in the immunodiffusion method above, was employed. Wells were punched with a Gelman gel punch (a 1.5 mm well for the antigen and a 1 mm trough for the antibody). The antigen plug was removed by vacuum and 2 ul of antigen (high-speed mouse liver supernatant) was added. Electrophoresis was carried out for 1 hour (3-6 mA per slide) using a Pharmacia EPS 500/400 power source. After electrophoresis, the antibody trough was removed by vacuum and 0.1 ml of rabbit anti-mouse PEPCK serum was added. The slide was incubated overnight at 25 C and read as in the immunodiffusion method above.

WESTERN BLOT:

This was performed according to the procedure of Burnette (34) which was adapted from that of Towbin (157). Gels were prepared as in the SDS-PAGE section above, except they were electrophoresed overnight at 4 mA and left

unstained. Half of the SDS-PAGE contained samples and the other half contained molecular weight markers to monitor the transfer of proteins onto the nitrocellulose. Transblot materials were assembled in a plastic container filled with degassed electrophoretic buffer (20mM Tris-base, 150 mM glycine, 20% (w/v) methanol, pH 8.3), in the following sequence. Bottom plastic holder, filter pad, two pieces of filter paper, nitrocellulose sheet (Schleicher & Schuell, cat. # BA85), SDS-PAGE gel, two pieces of filter paper, filter pad, and top plastic holder. Care was taken to avoid entrapment of air bubbles in any layer of assembly. The assembled material was quickly transferred to an electrophoretic chamber (Hoeffer TE 50 Transfer Electrophoretic Cell) which had been previously filled with electrophoretic buffer. The nitrocellulose sheet was positioned between the SDS-PAGE gel and the anode. Electrophoresis was run at 4°C for 2 hours at 50 V. The transblot apparatus was dismantled and the nitrocellulose was cut to separate samples from molecular weight markers. The nitrocellulose containing samples was heat-sealed in plastic (Sears counter craft, seal N-save) and stored at 4°C. The nitrocellulose containing molecular weight markers was stained in India ink as follows. Nitrocellulose was washed 4 times for 10 minutes each time in 250 ml wash buffer (PBS-TW: 0.15 M NaCl in 0.01 M Na_2HPO_4 / NaH_2PO_4, pH 7.2, containing 0.3% Tween 20) at 37°C. After each wash the nitrocellulose was thoroughly rinsed in water.

After the final wash, the nitrocellulose was stained with India ink (1 ul ink per ml PBS-TW) for 2 hours. Following confirmation of the transfer of proteins from the SDS-PAGE gel to the nitrocellulose, the stored nitrocellulose containing samples was saturated by incubation in 15 ml of a 10% milk-Tris-saline (MTS) solution (10% Carnation instant nonfat dry milk, 10 mM Tris-Hcl, 0.9% NaCl, pH 7.4) at 40°C for 30 minutes under constant rocking. After protein saturation, the nitrocellulose was incubated in 15 ml 10% MTS containing 200 ul rabbit anti-mouse PEPCK serum for 90 min. at 25°C. Occassional handling and turning of the bag afforded maximum contact of the antibody with the antigen. Following this incubation, the nitrocellulose was washed in 200 ml Tris-saline without milk for 10 minutes at 25°C, then in 200 ml Tris-saline containing 0.05% NP-40 for 10 minutes at 25°C, and finally in 200 ml Tris-saline for 10 minutes at 25°C. Following this wash, the nitrocellulose was incubated in 15 ml MTS containing 3 ul of ^{125}I-protein A, 3.7 x 10^6 cpm, (kindly provided by Dr. H. Bose, Dept. of Micro., U.T. Austin). Incubation proceeded at 25°C for 1 hour after which the nitrocellulose was blot dried and then air dried for 1-2 hours. The nitrocellulose was sealed in Saran wrap and autoradiographed in an intensifying screen exposed at -70°C to Kodak XR film.

PHOSPHOENOLPYRUVATE CARBOXYKINASE ASSAY:

Method 1: Essentially that of Colombo et al (41). This method was used to monitor the presence of PEPCK during its purification. In the reaction, PEPCK catalyzes the carboxylation of phosphoenolpyruvate (PEP) to oxaloacetic acid (OAA) which is subsequently converted to malic acid by malic dehydrogenase (MDH) with concomitant oxidation of NADH. The disappearance of NADH is followed at 340 nm as a decrease in absorbance over time. Purification of PEPCK was completed through the desalting step before assaying for activity due to the presence of NADH oxidizing activities in crude liver supernatants. The reaction mix contained:

56 mM HEPES buffer, pH 7.0-7.2
1 mM dithiothreitol (DTT)
2 mM $MnCl_2 \cdot 4H_2O$
0.2 mM NADH
22 units malate dehydrogenase (MDH)
2.3 mM inosine 5'-diphosphate (IDP)
2.5 mM phosphoenolpyruvate (PEP)

The reaction was run at 25°C by adding 0.7 ml of the reaction mix, less IDP and PEP, to a cuvette. Then 0.1 ml of sample containing PEPCK was added and the small decrease in absorbance was recorded. The reaction was started by the addition of 0.2 ml IDP/PEP and the decrease in absorbance was again recorded. The enzyme activity was calculated as follows:

$$U/ml = \frac{[(\Delta A_{+IDP} - \Delta A_{-IDP})/min] \text{ (rxn vol in ml)}}{\text{(sample vol in ml)}[6.23\text{(absorption coefficient)}]}$$

One unit is defined as that amount of enzyme that catalyzes the formation of one micromole product per minute at 25°C.

Method 2: This is a modification of the procedure of Ballard and Hanson (5) and Chang and Lane (36). This assay was used to measure the activity of PEPCK in crude liver supernatants. Livers were prepared according to the 'purification of PEPCK' method discussed earlier. The reaction is essentially that of the Colombo assay discussed above, except the carboxylation of PEP involved assaying the incorporation of radiolabeled $^{14}CO_2$ and the reaction mix contained 50 mM $KHCO_3$ containing 2 uCi $NaH^{14}CO_3$ (ICN # 17441-H), 100 mM imidazole buffer, pH 6.9, and 2.5 mM NADH. The reaction mixture was kept on ice in appropriately labeled 13 x 100 mm test tubes until the reaction was initiated. To begin the reaction, the tubes were prewarmed to 37°C for 3 minutes. At timed intervals, 50 ul of a 15% (w/v) high-speed liver supernatant was added. The reaction was terminated 15 minutes later by the addition of 25 ul of 15% (w/v) ice cold TCA. Each tube was immediately placed on ice. A control reaction less PEP and IDP was run with each assay and the result was subtracted from the sample result. Free $^{14}CO_2$ was removed from the reaction tube by bubbling unlabeled CO_2 through the

reaction solution for 30 minutes. After centrifugation for 2 minutes at 2,000 rpm, 0.4 ml of the reaction solution was counted in a total volume of 6.0 ml complete counting cocktail (RPI, Research Products International Inc., cat. # 11156), for 5 minutes on a scintillation counter. One unit of activity is defined as that amount of enzyme that catalyzes the fixation of one micromole of $NaH^{14}CO_3$ per minute at 37°C and was calculated as follows:

$$U/ml = \frac{\text{incorporated cpm as stable } ^{14}\text{C-malate}}{(\text{umole } NaH^{14}CO_3)(\text{rxn time, 15 min})(\text{ml sample, 0.05 ml})}$$

Specific activity was calculated as mU/mg protein.

Method 3: Enzyme linked immunosorbant assay (ELISA) was used to measure the amount of PEPCK in high-speed mouse liver supernatants. In this way a comparison between the amount of enzyme and activity of enzyme (method-2) can be made. The ELISA procedure used is an adapted version of that of Tsang et al (158). Plastic, 96 well, microtiter plates (Dynatech Laboratories cat. # 011-010-3650) were prewashed in 95% ethanol and rinsed in distilled water. After drying they were wrapped in plastic wrap and stored at 25°C until use . Upon use the wells were coated with 200 ul of 5 ug/ml rabbit anti-mouse PEPCK IgG in sensitizing buffer (0.05 M Tris, 2.0 mM EDTA, 0.3 M KCl, ph 8.0). Coating of antibody occurred by incubating at 37°C for 1 hour and then overnight

at 4°C. After coating the plates were washed 3 times for 3 minutes each with wash buffer (PBS-TW, 0.01 M Na_2HPO_4/NaH_2PO_4, 0.15 M NaCl, pH 7.2, with 0.3% Tween 20). Between each wash the plates were shaken dry and then blot dried on a layer of paper towells. To ensure complete saturation of all binding sites in the wells, the plates were then incubated overnight at 4°C with 200 ul of 0.5% Carnation nonfat dry milk in sensitizing buffer. Plates were washed as above. After the final wash, 200 ul of a 1:40 mouse liver homogenate in PBS-TW was added to the appropriate wells and the plates were incubated for 1 hour at 37 C. Plates were then washed as above. The enzyme was then sandwiched by the addition of 200 ul rabbit anti mouse PEPCK IgG:horseradish peroxidase (HRP) conjugate in PBS-TW made 0.01% with respect to thimerosal. Plates were incubated for 1 hour at 37°C then washed as before. During the final wash, fresh substrate was prepared as follows. To a scrupulously clean 100 ml graduated cylinder was added 10 ug o-phenylenediamine (OPD), dissolved in 1 ml of absolute methanol. This was taken to a volume of 100 ml to which 0.1 ml of a 3% hydrogen peroxide solution was added. After the final wash, 200 ul of this OPD substrate was added to each well. The conjugated HRP reduces H_2O_2 to 1/2 O_2 resulting in the subsequent oxidation of OPD and formation of an orange-yellow chromagen. The reaction was allowed to proceed at room temperature for 30 minutes, after which time it was terminated with 25 ul of 8 N H_2SO_4. The absorbance of the

plate wells were then read at 490 nm using an automatic microplate reader (Dynatech MR 600). A control with 5 ug/ml preimmune serum coated to the bottom of the wells was run with each assay and its absorbance was subtracted from that of the samples.

PREPARATION OF RABBIT ANTI-MOUSE PEPCK IgG:HORSERADISH PEROXIDASE CONJUGATE:

The procedure of Boorsma and Streefkerk (27) was employed. Horseradish peroxidase (HRP), 8 mg, was dissolved in 2 ml distilled, deionized water and 0.4 ml of freshly made 0.1 M $NaIO_4$ (periodate). This solution was stirred at 25 C for 20 minutes, during which time the periodate oxidized the aldehyde groups of HRP. After 20 minutes the oxidation was terminated by the addition of 3 drops of ethylene glycol. This solution was stirred at 25°C for 5 minutes. The solution was then desalted through a 2 x 12 cm Sepahadex G-25 fine column equilibrated with 0.001 M sodium acetate buffer, pH 4.2. The brown colored fractions were collected and pooled. To this was added 10 mg IgG (purified rabbit anti-mouse PEPCK antibody). The primary amines of the antibody reacted with the aldehyde groups of HRP. This conjugate solution was brought to pH 9.0-9.5 by adding 1 M Na_2HCO_3/ NaH_2CO_3, pH 9.5, in a dropwise fashion. After stirring for 2 hours at 25°C, 0.2 ml of freshly made NaBH (0.4 mg/ml) was added and the solution was incubated at 4°C for 2 hours.

This resulted in the reduction of any unreacted aldehyde groups. the conjugate solution was then dialyzed overnight against phosphate buffered saline (PBS), pH 7.0-7.2. The dialysate was stored at -20°C in aliquotes containing 0.01% thimerosal until use.

PROTEIN ASSAY:

The method of Bradford (29) was used. The absorbance of acidic Coomassie brilliant blue G-250 shifts from 465 to 595 nm when bound by protein and the amount of binding is directly proportional to color intensity. Concentrated dye reagent (BioRad cat. # 500-006) was diluted 1:4 in distilled water, filtered through Whatman no. 1, and stored in glass at 25°C. To 5.0 ml of diluted dye reagent was added 0.1 ml appropriate diluted protein (eg, a 1:10 dilution of a 15% (w/v) high speed mouse liver supernatant). This was mixed by gentle inversion and allowed to set at least 15 minutes, but no longer than 1 hour, before reading on a Beckman uv/vis spectrophotometer at 595 nm against a diluted dye:distilled water blank. Standard proteins were run with each assay to establish a standard curve from which protein concentrations (mg/ml) were read.

RESULTS

EFFECT OF STIMULATING THE RETICULOENDOTHELIAL SYSTEM (RES) ON SENSITIZATION OF MICE TO ENDOTOXIN: Benacerraf (9) showed that mice were sensitized to the lethal effects of LPS upon prior stimulation of their reticuloendothelial system (RES) with zymosan. Since the biological effects of LPS are mediated by leukocytes, primarily macrophages (111), it seems likely that a proliferation of these cells in the spleen and other RES tissue could result in this acute sensitization to LPS. We primed the RES of CFW mice with a heat-killed, formalin-fixed preparation of Corynebacterium parvum prior to challenge with toxin endotoxin (LPS) or radiodetoxified endotoxin (RD-LPS). These results are shown in Table 1. With a sufficient dose-rate of LPS, C. parvum primed mice began to die as early as 2 hours post-challenge. These were convulsive deaths characterized by bloody diarrhea, hematuria, proteinuria and splenomegaly. Eight hours after the administration of LPS the LD_{50} for mice whose RES had been stimulated was approximately 5 ug for LPS and 20 ug for RD-LPS. No deaths were observed during this same time when unprimed mice were challenged with as much as 500 ug of LPS. These data show the lethal activity of detoxified LPS (RD-LPS) during biological conditions which optimize the lethal effects of endotoxin. However, even under such conditions RD-LPS remains less toxic than LPS. The exact chemical reason for a decrease

Table 1: Sensitization of mice primed with Corynebacterium parvum to LPS or RD-LPS.

Treatment	Dose level	% Mortality at time (hours) Cumulative deaths/total 4	6	8
Unprimed + LPS[a]	500 ug	0 0/13	0 0/13	0 0/13
Primed + LPS[b]	20 ug	47 7/15	80 12/15	100 15/15
	5 ug	27 3/11	54 6/11	54 6/11
	0.8 ug	0 0/12	25 3/12	42 5/12
Primed + RD-LPS[c]	20 ug	15 3/20	45 9/20	50 10/20
	5 ug	0 0/12	33 4/12	42 5/12
	0.8 ug	0 0/12	8 1/12	8 1/12

[a] Normal mice given 500 ug LPS (iv) derived from S.typhimurium

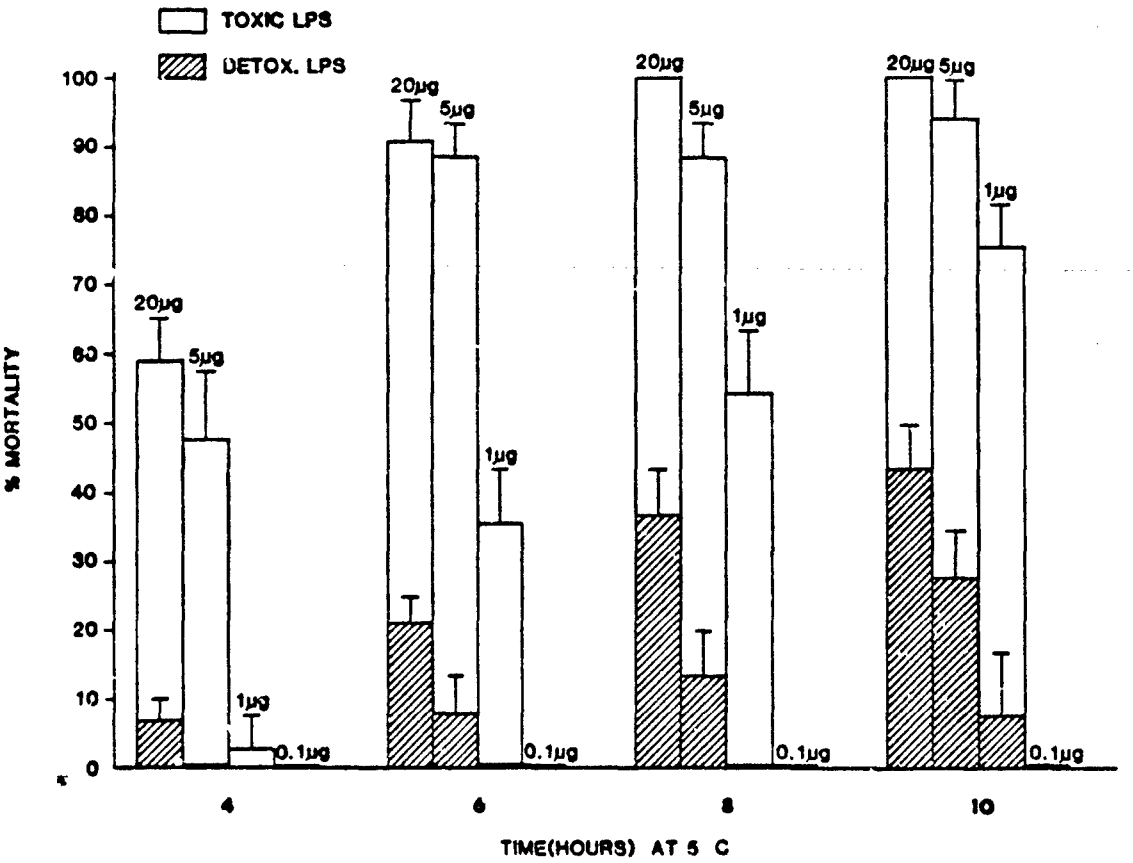
[b] Mice injected with 700 ug of heat-killed C. parvum (iv) and given various doses of LPS (iv) 6 days later.

[c] Mice injected with 700 ug of heat-killed C. parvum (iv) and given various doses of RD-LPS (iv) 6 days later.

in the toxicity of RD-LPS is unknown, although irradiation does result in a decrease in the keto-deoxyoctonate (KDO) and glucosamine groups (23). Loss of these would render the molecule more hydrophobic and potentially less reactive with those cells of the RES which produce mediators involved in the pathophysiologic effects of LPS. These biological differences are even more speculative than the chemical ones and further studies are needed to resolve the animals response to LPS versus RD-LPS.

EFFECT OF HEAT AND COLD STRESS ON SENSITIZATION OF MICE TO ENDOTOXIN: It is well known that mice subjected to temperature stress survive poorly when treated with LPS (13, 124,125). We observed the effect of RD-LPS in mice which were housed at 5°C and 37°C and compared this toxicity with that of toxic LPS. In mice exposed to 5°C (Figure 1), LPS was more lethal than RD-LPS. At 10 hours the LD_{50} for LPS was 0.5 ug. This is a 700-fold increase in toxicity over animals poisoned at 25°C where the LD_{50} is 345 ug at 24 hours after challenge with LPS. The LD_{50} for RD-LPS was approximately 20 ug at 10 hours of cold stress. This is a 50-fold increase in toxicity over animals poisoned at 25 C°where the LD_{50} is about 1000 ug at 24 hours (23). Though RD-LPS is less toxic than LPS in mice exposed to cold, even its diminished toxicity is significant when compared to cold-stressed mice receiving saline as a placebo. None of the unpoisoned mice

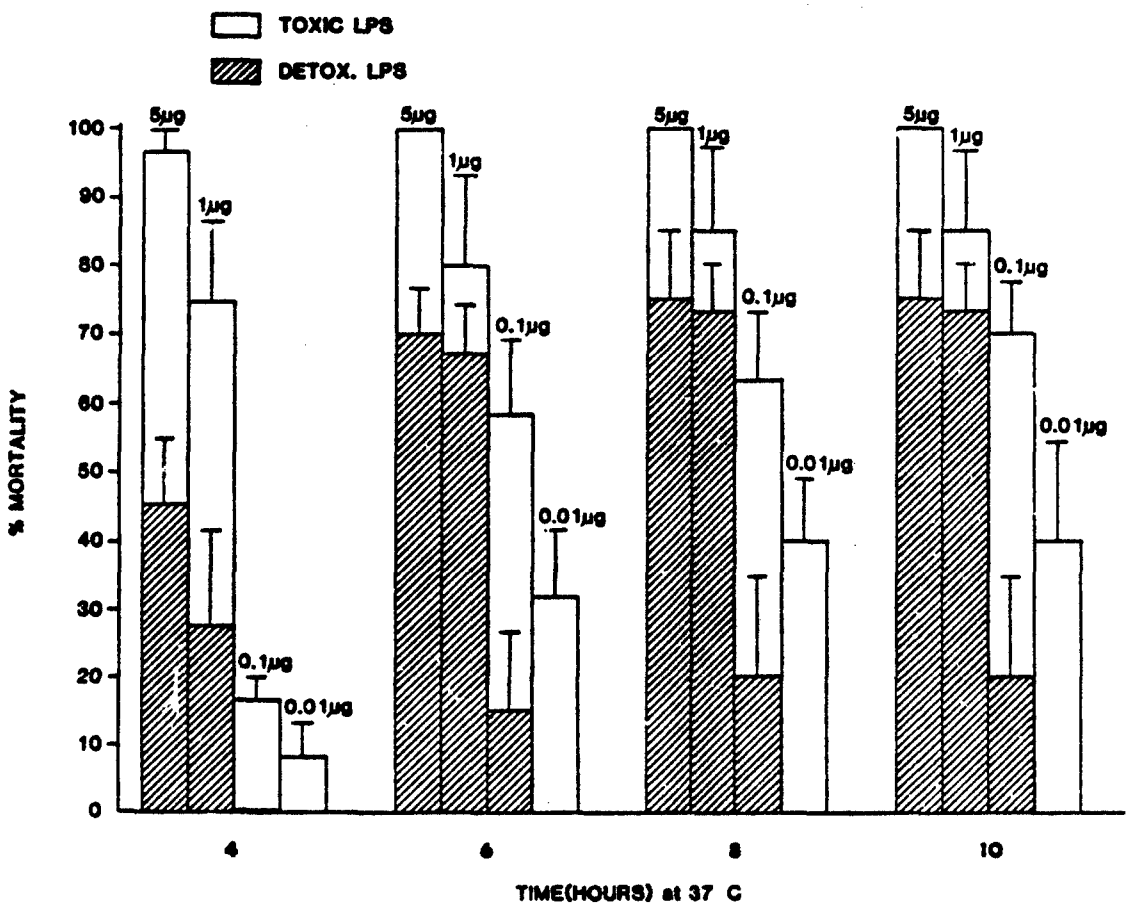
Figure 1: Effect of cold stress on sensitization of mice to an intravenous injection of LPS (clear bars) or radiodetoxified LPS (shaded bars). Percent mortality was calculated and plotted along the ordinate against various times (hours) after challenge along the abscissa. Fasted mice were housed at 5°C, singly, without bedding, in a walk-in refrigerator. Mortality rates are calculated from at least 3 separate experiments, each with at least 5 mice per group.


TOXIC LPS
DETOX. LPS
% MORTALITY
100
90
80
70
60
50
40
30
20
10
0
20µg
5µg
1µg
0.1µg
4
6
8
10
TIME(HOURS) AT 5 C

died when exposed to 5°C for 10 hours. The LD_{50} was calculated at 10 hours in all temperature-stress experiments because there were significant mortality rates in mice treated with LPS while those given only saline were relatively unaffected within this time span (see legend to Figure 3).

During heat stress the decrease in the LD_{50} for RD-LPS and LPS is even more dramatic than during cold stress. Figure 2 shows that at 10 hours of incubation at 37°C the LD_{50} for LPS is 0.04 ug, or a 10,000-fold increase in toxicity over mice poisoned at 25°C. As in cold stress, RD-LPS is less lethal than LPS during heat stress. At 10 hours of incubation at 37°C the LD_{50} for RD-LPS is 0.5 ug. This is a 2,000-fold increase in toxicity relative to the LD_{50} for mice housed at 25°C. These data show that the potency of both LPS and RD-LPS are intensified during temperature stress and this is more dramatic at 37°C than at 5°C. The reason for increased sensitization at 37°C is unknown, but the animals response to LPS may differ in heat versus cold stress. It has been reported that B-endorphins, the endogenous opiates released during endotoxinemia, may regulate hyperthermia (82) but not hypothermia (137). Since naloxone antagonizes the effects of B-endorphins (52,64,81,82) and glucocorticoids afford protection against the lethal effects of LPS (12,14, 61,99), we tried to protect our temperature-stressed mice against the toxic effects of LPS with these two agents.

Figure 2: Effect of heat stress on sensitization of mice to an intravenous injection of LPS (clear bars) or radiodetoxified LPS (shaded bars). Percent mortality was calculated and plotted along the ordinate against various times (hours) after challenge along the abscissa. Fasted mice were housed at 37°C, 5 per cage, in a walk-in incubator. Mortality rates were calculated from at least 3 separate experiments, each with at least 5 mice per group.


TOXIC LPS
DETOX. LPS
% MORTALITY
100
90
80
70
60
50
40
30
20
10
0
5µg
1µg
0.1µg
0.01µg
4
6
8
10
TIME(HOURS) at 37 C

EFFECT OF HYDROCORTISONE OR NALOXONE IN PROTECTING MICE AGAINST LETHALITY DUE TO ENDOTOXIN DURING COLD AND HEAT STRESS: Hydrocortisone, given as a 50 mg/kg subcutaneous injection prior to LPS increased the survival rate of mice during cold or heat stress (Figures 3 and 4). Using an approximate LD_{70} dose of LPS (1 ug at 5°C and 0.1 ug at 37°C), 47% (16/34) and 33% (10/30) of the mice were dead when housed at 5°C or 37°C (Figures 3 and 4), respectively, at 10 hours after challenge with LPS plus hydrocortisone. At both temperatures this is about one-half the mortality rate seen in mice given LPS alone where 79% (26/33) of the mice died in the cold (Figure 3) and 73% (19/26) of the mice died in the heat (Figure 4) at 10 hours. In addition to increasing survival, hydrocortisone enabled mice to maintain a more normal body temperature (Figures 3 and 4, clear bars). At 10 hours LPS-poisoned mice had an average body temperature of 21°C in the cold and 40.2°C in the heat. LPS-poisoned mice given hydrocortisone maintained an average body temperature of 26°C and 39.6°C at 5°C and 37°C, respectively, at 10 hours. Regardless of the time of death after challenge with LPS, mice which died during cold stress exhibited a body temperature of about 20°C. This is almost 20°C cooler than normothermia. This wide fluctuation is not observed at 37°C. LPS-poisoned animals exposed to heat present with only a 2-3° C hyperthermic response at the time of death. This may indicate that regulation of hyperthermia is more critical

Figure 3: Ability of hydrocortisone or naloxone to protect mice housed at 5°C against lethality from 1 ug of LPS. Et = LPS (1 ug, iv). NX = naloxone (5 mg/kg, sc) plus LPS. HC = hydrocortisone (50 mg/kg, sc) plus LPS. C = normal mice. Percent mortality (dark bars) was calculated and colonic temperatures (light bars) were measured and plotted along the ordinate against various times (hours) after challenge plotted along the abscissa. Fasted mice were housed singly, without bedding, in a walk-in refrigerator. Mortality rates and colonic temperatures were calculated from at least 3 separate experiments, each with at least 5 mice per group.

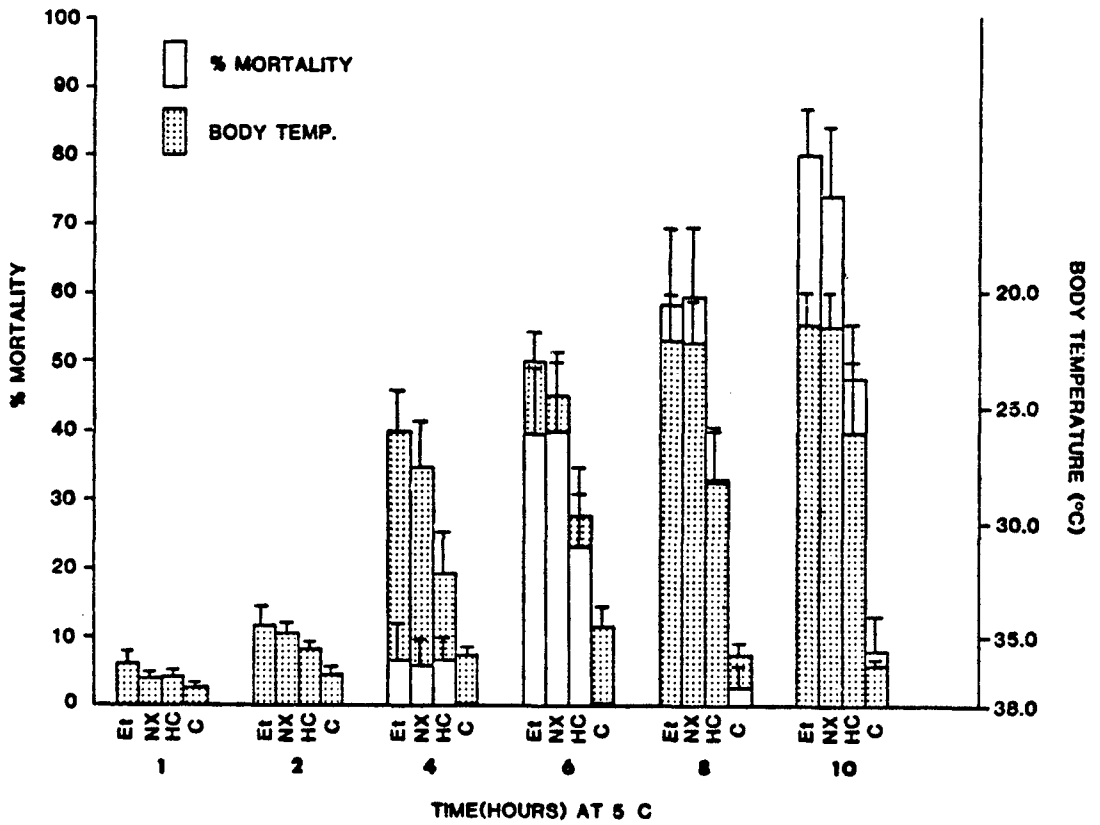
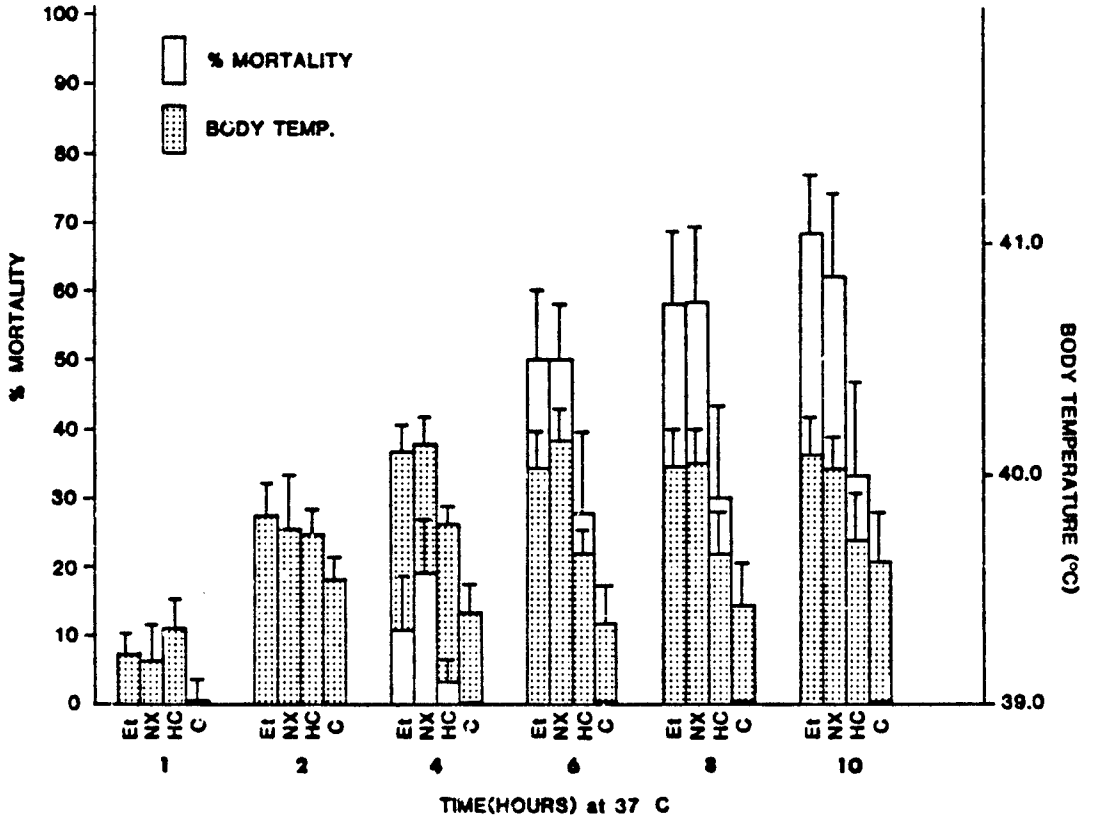

% MORTALITY
BODY TEMP.
% MORTALITY
BODY TEMPERATURE (°C)
100
90
80
70
60
50
40
30
20
10
0
20.0
25.0
30.0
35.0
38.0
Et NX HC C
1
2
4
6
8
10
TIME(HOURS) AT 5 C

Figure 4: Ability of hydrocortisone or naloxone to protect mice housed at 37°C against lethality from 0.1 ug of LPS. Et = LPS (0.1 ug, iv). NX = naloxone (5 mg/kg, sc) plus LPS. HC = hydrocortisone (50 mg/kg, sc) plus LPS. C = normal mice. Percent mortality (dark bars) was calculated and colonic temperatures (light bars) were measured and plotted along the ordinate against various times (hours) after challenge plotted along the abscissa. Fasted mice were housed 5 per cage in a walk-in incubator. Mortality rates were calculated and colonic temperatures were measured from at least 3 separate experiments, each with at least 5 mice per group.


% MORTALITY
BODY TEMP.
% MORTALITY
BODY TEMPERATURE (°C)
100
90
80
70
60
50
40
30
20
10
0
41.0
40.0
39.0
Et
NX
HC
C
1
2
4
6
8
10
TIME(HOURS) at 37 C

to the animals survival. It has been shown that B-endorphins regulate hyperthermia (82) but not hypothermia (64). It seemed reasonable then to study the effect of blocking endogenous B-endorphins with the opiate antagonist, naloxone, during temperature stress.

Naloxone, given as a 5 mg/kg subcutaneous injection 5 minutes prior to LPS, neither attenuated mortality nor cold-stress induced hypothermia or heat-stress induced hyperthermia. At 5°C mice given naloxone prior to LPS exhibited a mortality rate of 73% (27/37) and their average body temperature was 21°C at 10 hours (Figure 3). This is essentially the same as in mice treated with LPS alone where a mortality rate of 79% (26/33) and average body temperature of 21°C was observed at 10 hours. Similar parity was noted at 37°C (Figure 4). At 10 hours the mortality rate of mice treated with LPS alone or LPS plus naloxone were similar, 68% (19/26) and 62% (16/26), respectively. Mice treated with naloxone prior to challenge with LPS had an average body temperature of 40.1°C at 10 hours during heat stress and this is about the same as the average reading of 40.2°C taken in mice which received LPS alone.

From these studies it must be concluded that hydrocortisone increases the survival rate of endotoxinemic mice during both heat and cold stress. This is characterized by the animals ability to maintain a more normal body temperature. On the other hand, naloxone did not afford protection

or alter the animals hypothermic response in the cold or hyperthermic response in the heat. This suggests that B-endorphins do not contribute to the demise of LPS-poisoned animals during temperature stress and they have no clear role in long-term regulation of hyperthermia or hypothermia. However, during the early stages of heat stress, naloxone may have antagonized the ability of B-endorphins to regulate hyperthermia. At 4 hours, mice treated with LPS plus naloxone had a core temperature which was 0.3°C higher than mice treated with LPS alone (Figure 4). This increase in body temperature may either be biologically insignificant or else deregulation of hyperthermia may merely be a symptom of the animals response to LPS as the mortality rates for both groups of mice is the same at this time. Since very small dose-levels of LPS and RD-LPS are fatal for mice during stress to heat or cold and for mice whose RES has been stimulated, the ability of RD-LPS to elicit an RES derived serum factor which could sensitize mice to the conditions of temperature stress was tested.

EFFECT OF HEAT AND COLD STRESS ON SENSITIZATION OF MICE TO GLUCOCORTICOID ANTAGONIZING FACTOR: Couch et al (43) showed that mice were sensitized to the cold with serum collected 2 hours after LPS was given to mice whose RES was primed with zymosan. This same format was used to collect serum from mice whose RES had been activated 6 days earlier

with 700 ug of heat-killed Corynebacterium parvum. The serum was collected 2 hours after challenge with 25 ug of LPS or RD-LPS. It was abbreviated CpES for C. parvum endotoxin serum and CpdES for C. parvum detoxified endotoxin serum. Mice which were made tolerant to LPS were used in this experiment in order to test for the presence of a humoral factor and to negate the effects of any residual endotoxin remaining in the serum. At 5°C, CpES and CpdES are both very potent as early as 4 hours after administration (Figure 5). At 4 hours 93% (25/27) of the mice challenged with CpES and 90% (18/20) of the mice challenged with CpdES were dead. Control sera collected from C. parvum primed mice without LPS (CpS), from unprimed mice given LPS (ES) and from unprimed mice given RD-LPS (dES) were significantly less lethal. A control group of mice (column C in Figure 5) was also included to show the unresponsiveness of tolerant mice to a 10 x LD_{50} of LPS. Similar results were obtained during heat stress. At 37 C, CpES and CpdES killed 100% (25/25) and 88% (14/16), respectively, of the endotoxin tolerant mice 4 hours after challenge (Figure 6). The mortality rates for mice given control sera (CpS, ES, and dES) were dramatically less even at 8 hours after challenge.

These data indicate the presence of an LPS-induced serum mediator produced by cells of the RES which sensitizes endotoxin tolerant mice to cold and heat stress. Also, cells of the RES produce this mediator in response to either LPS or

Figure 5: Effects of cold stress on sensitization of mice, made tolerant to endotoxin, to glucocorticoid antagonizing factor (GAF). Percent mortality was calculated and plotted along the ordinate against various times (hours) after challenge plotted along the abscissa. Fasted mice were housed singly, without bedding in a 5°C walk-in refrigerator. All mice were rendered tolerant to endotoxin. C = mice given a 10x LD_{50} of LPS. CpS = sera collected 6 days after challenge with C. parvum. ES = sera collected 2 hours after challenge with 25 ug LPS. dES = sera collected 2 hours after challenge with 25 ug RD-LPS. CpES = sera collected 2 hours after challenge with 25 ug LPS in mice primed 6 days earlier with C. parvum. CpdES = sera collected 2 hours after challenge with 25 ug RD-LPS in mice primed 6 days earlier with C. parvum.

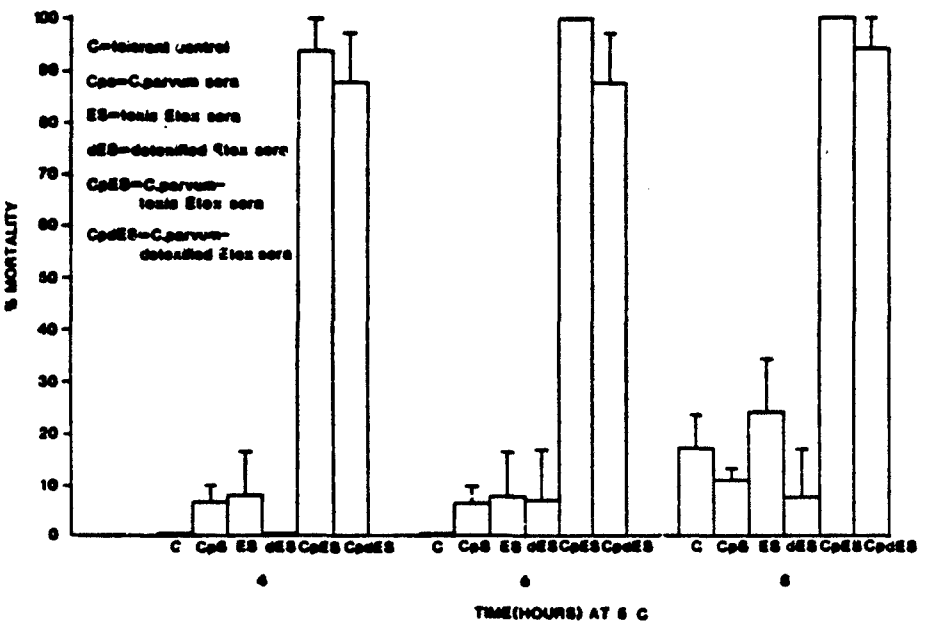
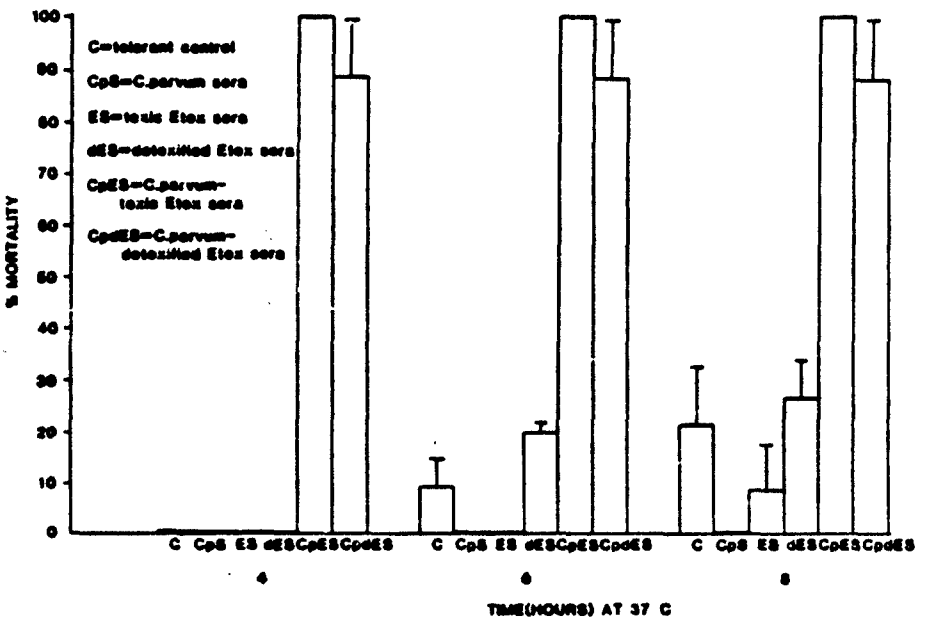

C=tolerant control
Cps=C.parvum sera
ES=toxin Etx sera
dES=detoxified Etx sera
CpES=C.parvum-toxin Etx sera
CpdES=C.parvum-detoxified Etx sera
% MORTALITY
C Cps ES dES CpES CpdES
4
6
8
TIME(HOURS) AT 6 C

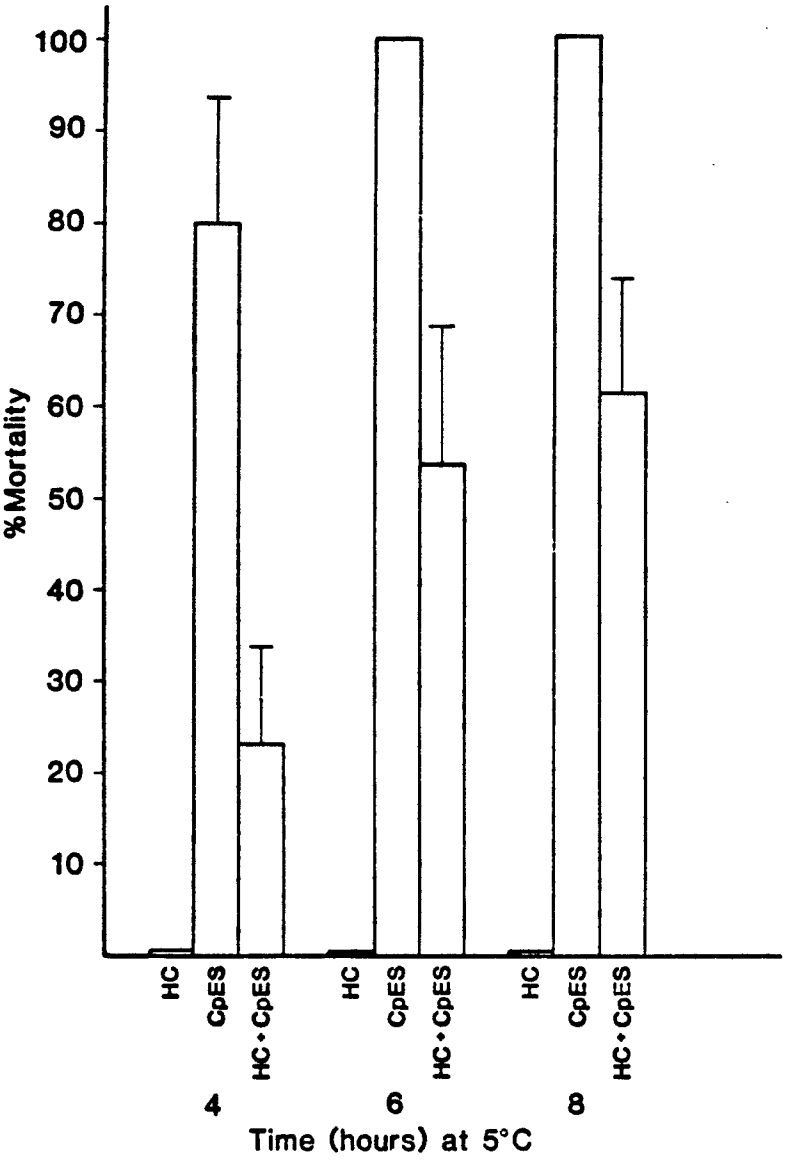
Figure 6: Effects of heat stress on sensitization of mice, made tolerant to endotoxin, to glucocorticoid antagonizing factor (GAF). Percent mortality was calculated and plotted along the ordinate against various times (hours) after challenge plotted along the abscissa. Fasted mice were housed in a 37°C walk-in refrigerator. All mice were rendered tolerant to endotoxin. C = mice given a 10x LD_{50} of LPS. CpS = sera collected 6 days after challenge with C. parvum. ES = sera collected 2 hours after challenge with 25 ug of LPS. dES = sera collected 2 hours after challenge with 25 ug of RD-LPS. CpES = sera collected 2 hours after challenge with 25 ug of LPS in mice primed 6 days earlier with C. parvum. CpdES = sera collected 2 hours after challenge with 25 ug of RD-LPS in mice primed 6 days earlier with C. parvum.


C=tolerant control
CpS=C.parvum sera
ES=toxic Etox sera
dES=detoxified Etox sera
CpES=C.parvum-toxic Etox sera
CpdES=C.parvum-detoxified Etox sera
% MORTALITY
100
90
80
70
60
50
40
30
20
10
0
C CpS ES dES CpES CpdES
4
6
8
TIME(HOURS) AT 37 C

RD-LPS. This is shown in figures 5 and 6 where lethality due to CpdES essentially parallels that of CpES from 4 to 8 hours of cold or heat stress. Because this serum is known to antagonize the effects of exogenous hydrocortisone in vivo (111, 112,114) and in rat hepatoma cells in vitro (65,66), the mediator was called 'glucocorticoid antagonizing factor (GAF)'. Inhibition of the adrenal cortical response during heat and cold stress is the most probable explanation for lethality due to GAF as adrenalectomized animals survive poorly during temperature stress (74) and this can be reversed by exogenous corticosteroids (74).

EFFECT OF HYDROCORTISONE IN PROTECTING MICE AGAINST LETHALITY DUE TO GLUCOCORTICOID ANTAGONIZING FACTOR (GAF) DURING COLD AND HEAT STRESS: GAF-rich serum (CpES and CpdES) is lethal for mice placed in the cold or heat (Figures 5 and 6); furthermore, exogenous hydrocortisone protects mice poisoned with LPS against lethality due to LPS during temperature stress (Figures 3 and 4). Therefore, it seemed necessary to try and protect temperature-stressed mice against lethality due to GAF by giving them exogenous hydrocortisone. At 5°C (Figure 7) hydrocortisone afforded partial protection to mice poisoned with CpES (GAF-rich serum). At 4 hours, 80% (8/10) of the mice given CpES were dead while only 23% (3/13) of the mice given 50 mg/kg of hydrocortisone plus CpES had died. The mortality rate of the hydrocortisone treated group

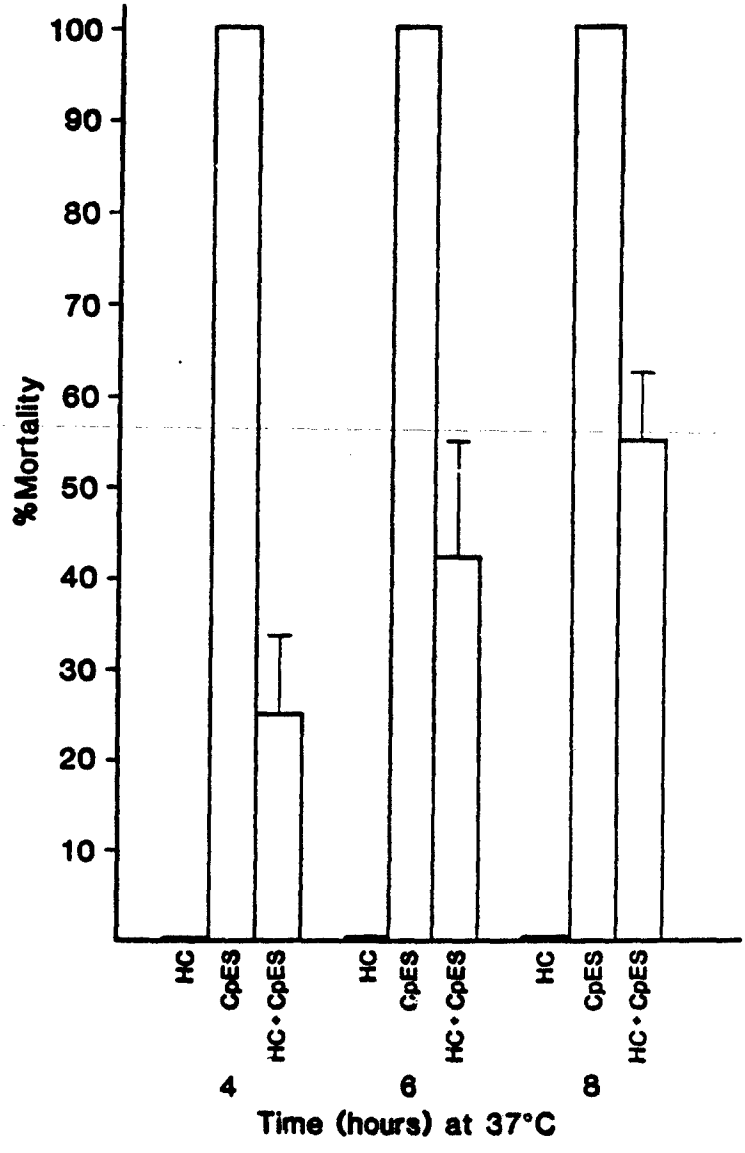
Figure 7: Protective effect of hydrocortisone on survival of tolerant mice subjected to GAF-rich serum during stress to cold. Percent mortality was calculated and plotted along the ordinate against various times (hours) after challenge plotted along the abscissa. HC = hydrocortisone given sc, 50 mg/kg (6 mice per group). CpES = C. parvum endotoxin serum given iv, 0.4 ml (10 mice per group). HC + CpES = hydrocortisone given sc, 50 mg/kg, 2 hours prior to CpES given iv, 0.4 ml (13 mice per group). Fasted mice were housed, singly, without bedding in a walk-in refrigerator at 5°C.


100
90
80
70
60
50
40
30
20
10
% Mortality
HC
CpES
HC + CpES
HC
CpES
HC + CpES
HC
CpES
HC + CpES
4
6
8
Time (hours) at 5°C

rose to 61% (8/13) at 8 hours, but this is still significantly less than the number of deaths seen in mice which were not pretreated with the hormone. At 37°C (Figure 8), similar results were observed. All of the mice receiving CpES were dead at 4 hours (25/25) compared to a 26% (7/27) mortality rate for the group pretreated with hydrocortisone. As with exposure to 5°C, stress for 8 hours at 37°C showed similar results. The mice without hormone treatment had a mortality rate about twice that of mice which did receive hydrocortisone, 100% (25/25) and 52% (17/27), respectively. Thus hydrocortisone, given 2 hours prior to serum (CpES) containing enhanced levels of GAF notably increased the survival rate of mice stressed by cold and by heat. This is significant as it indicates that not only can GAF antagonize the effects of hydrocortisone, but the hormone can also antagonize the effects of GAF.

EFFECT OF SENSITIZING MICE TO THE LETHAL EFFECTS OF LPS WITH A PRIOR DOSE OF LPS OR RD-LPS: This experiment shows that mice which are stressed with a small dose of LPS or detoxified LPS are sensitized to a second sublethal dose of LPS 6 hours later. Figure 9 shows that administration of 50 ug LPS followed 6 hours later with 150 ug of LPS results in a mortality rate of 53% (16/30) at 24 hours and 77% (23/30) at 48 hours after challenge with the second dose. Also, 50 ug of RD-LPS sensitizes mice to this second injection of LPS as 58%

Figure 8: Protective effect of hydrocortisone on survival of tolerant mice subjected to GAF-rich serum during stress to heat. Percent mortality was calculated and plotted along the ordinate against various times (hours) after challenge plotted along the abscissa. HC = hydrocortisone given sc, 50 mg/kg (7 mice per group). CpES = C. parvum endotoxin serum given iv, 0.4 ml (10 mice per group). HC + CpES = hydrocortisone given sc, 50 mg/kg, 2 hours prior to CpES given iv, 0.4 ml (27 mice per group). Fasted mice were housed, 5 per cage in a walk-in incubator at 37°C.


100
90
80
70
60
50
40
30
20
10
% Mortality
HC
CpES
HC + CpES
HC
CpES
HC + CpES
HC
CpES
HC + CpES
4
6
8
Time (hours) at 37°C

(10/17) and 70% (12/17) of these mice were dead at 24 and 48 hours, respectively. Initial stress of the mouse must be caused by the first 50 ug dose-level because the combined total of both dose levels (ie, 200ug) results in a mortality rate of only 7% (1/15) at both 24 and 48 hours after challenge (Figure 9).

Exogenous hydrocortisone, 50 mg/kg, repressed this lethality and its effect was more intense when given concurrent with the 50 ug priming dose of LPS than when given concurrent with the second 150 ug dose of LPS 6 hours later. Mice given hydrocortisone concurrent with the priming dose of LPS exhibited a mortality rate of 15% (4/26) at 24 hours and 23% (6/26) at 48 hours (Figure 9). This is significantly less than the mortality rates of 58% (10/17) and 70% (12/17) at 24 and 48 hours, respectively, seen in mice which did not receive hydrocortisone. However, when the same dose of the hormone was given 6 hours after the priming dose of LPS and concurrent with the second dose of LPS, it was less protective. The mortality rates for these mice were 38% (5/13) at 24 hours and 54% (7/13) at 48 hours (Figure 9).

These data suggest that the priming dose of LPS stimulates the production of a glucocorticoid antagonizing factor which impairs the effects of exogenous hydrocortisone and sensitizes mice to an otherwise sublethal dose of LPS given 6 hours later. This initial glucocorticoid antagonizing activity can be opposed when hydrocortisone is given with

Figure 9: Sensitization of mice to the lethal effects of LPS following a small dose of LPS or RD-LPS 6 hours earlier. Percent mortality was calculated and plotted along the ordinate against various treatments as shown along the abscissa. The clear bar in a given treatment group represents an elapse of 24 hours and the shaded bar an elapse of 48 hours. All LPS injections were given iv in a total volume of 0.2 ml. Hydrocortisone was given in a dose of 50 mg/kg, sc, in a total volume of 0.2 ml.

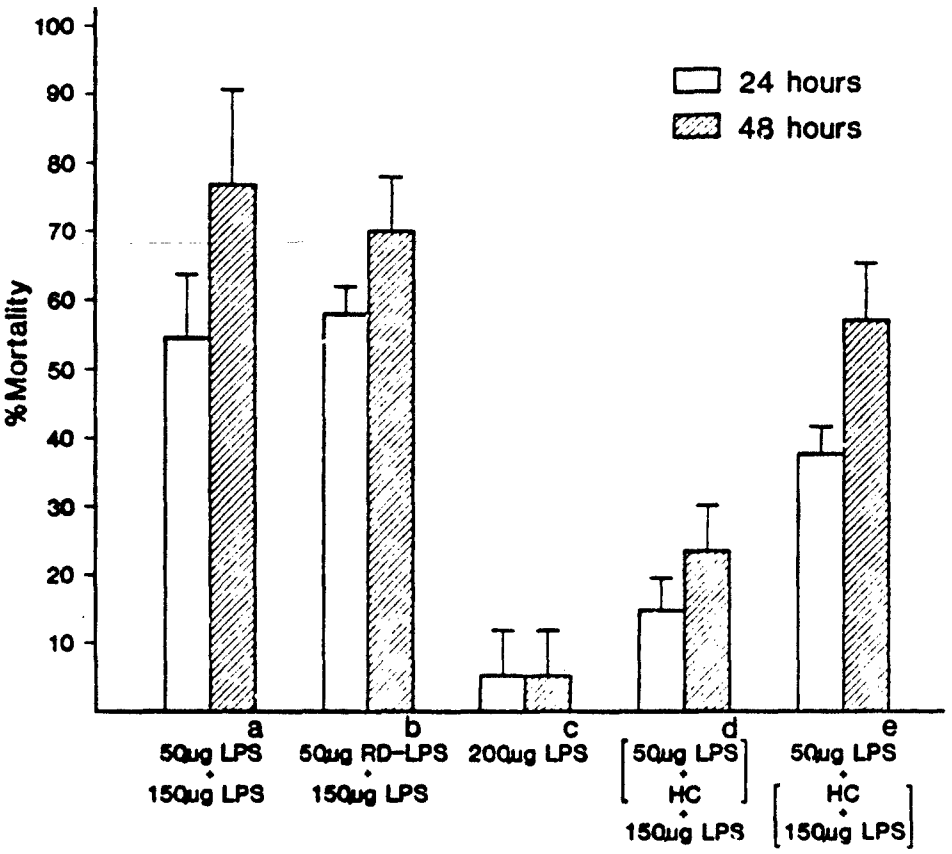
a
50 ug LPS given 6 hours prior to 150 ug LPS (30 mice/group).

b
50 ug RD-LPS given 6 hours prior to 150 ug LPS (17 mice/group).

c
200 ug LPS given as a bolus control dose (15 mice/group).

d
50 ug LPS given concurrent with 50 mg/kg of hydrocortisone 6 hours prior to 150 ug LPS (13 mice/group).

e
50 ug LPS given 6 hours prior to 150 ug LPS given concurrent with 50 mg/kg of hydrocortisone (13 mice/group).


100
90
80
70
60
50
40
30
20
10
%Mortality
24 hours
48 hours
a
50ug LPS
+
150ug LPS
b
50ug RD-LPS
+
150ug LPS
c
200ug LPS
d
[50ug LPS
+
HC
+
150ug LPS]
e
50ug LPS
+
HC
+
[150ug LPS]

the priming dose of LPS. Since GAF has been shown to predispose mice to the lethal effects of LPS (43), it seems reasonable to suggest that the priming dose of LPS elicits the production of GAF which renders the animal susceptible to a second exposure to LPS. This is further support for the requirement of an adrenal response during stress and the ability of GAF to block this response.

EFFECT OF TOURNIQUET SHOCK ON SENSITIZATION TO LPS AND GAF: In addition to heat stress, cold stress, and stress induced by priming mice with a sublethal dose of LPS 6 hours prior to challenge, the effect of stress due to tourniquet shock on sensitization of mice to LPS or GAF was tested. Tourniquet shock was described in 1943 when Rosenthal (136) showed maximum lethality in mice after the tourniquet had been applied for 2 hours. If tourniquet stress could be induced without the associated lethality, it would provide a system by which mice could be sensitized to the lethal effects of LPS or serum rich in GAF (CpES). The application of the tourniquet is discussed in detail in the materials and methods section of this thesis.

Figure 10 shows that at 24 hours all of the mice (6/6) stressed by tourniquet application for 2 hours were dead. However, when the tourniquet was apllied for only 1 hour, none of the mice (0/13) had died. Therefore , a 1 hour application time was selected as a means of stress. When LPS

was given at the time of tourniquet application, the mice survived poorly (Figure 10). At 24 hours, all of the mice died (10/10) when given 25 ug of LPS, 91% (10/11) were dead after receiving 5 ug of LPS, and 1 ug of LPS was lethal for 48% (10/21) of the mice (Figure 10, clear bars). Exogenous hydrocortisone (50 mg/kg) provided partial protection against lethality due to endotoxin when it was given concurrently with LPS at the time of the tourniquet application. At 24 hours the mortality rates for these mice were 21% (3/14) and 43% (6/14) with an LPS dose of 1 ug or 5 ug, respectively.

Mice placed under the stress of tourniquet shock were also highly sensitive to the lethal effects of serum containing GAF (CpES, Figure 10). By 24 hours, all of the mice (10/10) treated with CpES had died. Serum which does not contain appreciable levels of GAF (ES for endotoxin serum and CpS for C. parvum serum) were essentially nontoxic, exhibiting mortality rates of 7% (1/14) and no mortality (0/7), respectively. In sharp contrast to protection against LPS, hydrocortisone did not protect mice during tourniquet stress against lethality due to CpES. All mice (8/8) treated with 50 mg/kg of hydrocortisone concurrent with 0.4 ml of CpES at the time of application of the tourniquet were dead at 24 hours. These data indicate that hydrocortisone can afford some protection when given prior to the production and/or release of GAF, but pre-existing GAF completely blocks protection afforded by this hormone.

Figure 10. Effect of tourniquet shock on sensitization of mice to the lethal effects of LPS or GAF. Percent mortality was calculated and plotted along the ordinate against various treatments shown along the abscissa. All LPS injections were given iv in a total volume of 0.2 ml. All serum injections were given iv in a total volume of 0.4 ml. Hydrocortisone was given as a 50 mg/kg dose. sc in a volume of 0.2 ml. In the LPS treatment group, the clear bars represent LPS given alone and the shaded bars represent LPS given concurrently with hydrocortisone.

a
Tourniquet application for 2 hours (6 mice/group).

b
Tourniquet application for 1 hour (13 mice/group).

c-e
LPS given at the time of tourniquet application (for c, 7 mice/ group; for d, 11 mice/group; for e, 21 mice/group).

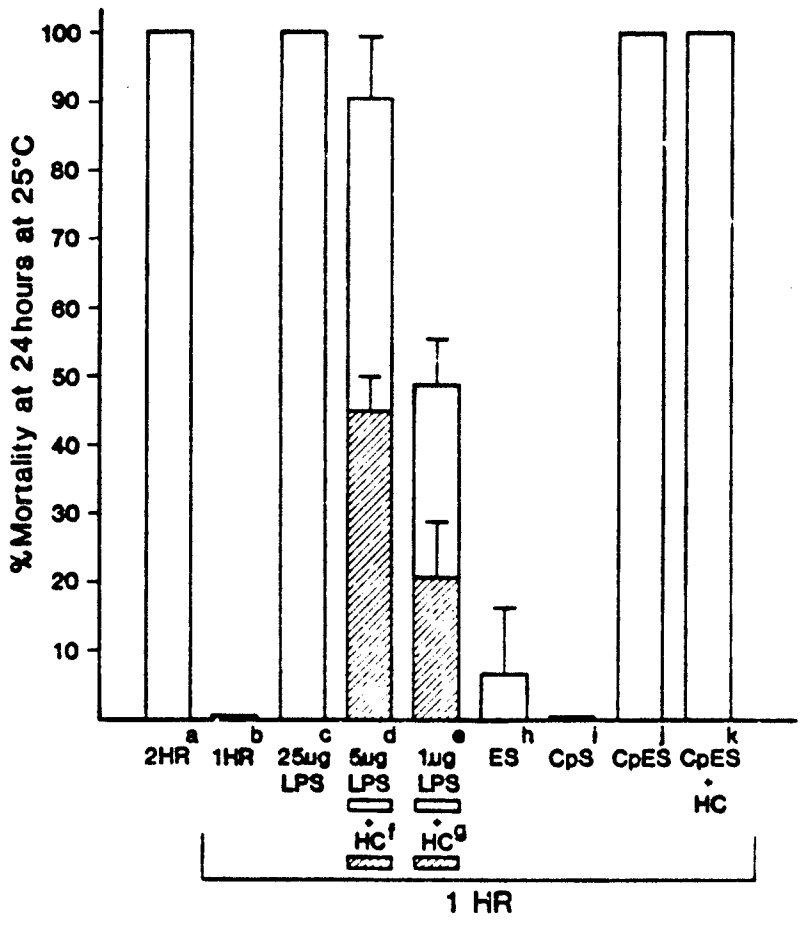
f,g
LPS given concurrently with hydrocortisone at the time of tourniquet application (14 mice/group).

h
Serum collected 2 hours after challenge with 25 ug LPS. Given at the time of tourniquet application (14 mice/group).

i
Serum collected 6 days after challenge with C. parvum. Given at the time of tourniquet application (7 mice/group).

j
Serum collected 2 hours after challenge with 25 ug LPS given to mice treated 6 days earlier with C. parvum (8 mice/group).

k
As in j. Given concurrent with hydrocortisone (8 mice/group).


% Mortality at 24 hours at 25°C
100
90
80
70
60
50
40
30
20
10
a
2HR
b
1HR
c
25µg
LPS
d
5µg
LPS
+
HC f
e
1µg
LPS
+
HC g
h
ES
i
CpS
j
CpES
k
CpES
+
HC
1 HR

PURIFICATION OF PEPCK AND ACQUISITION OF ANTI-PEPCK ANTIBODY:

The purification of this enzyme is detailed in the Material and Methods section of this thesis. Enzyme activity was measured at various steps of the purification process as shown in Table 2. Precipitation between 45% and 65% ammonium sulfate saturation resulted in a 3-fold purification of the enzyme as seen in the specific activity column in Table 2. Proteins precipitated by ammonium sulfate were then desalted by passage through a gel filtration column in which proteins larger than 6 kilodaltons eluted with the void volume while the passage of smaller proteins through the column was delayed. This resulted in an additinal 1.5-fold purification of PEPCK. Further purification was achieved by ion exchange chromatography. Proteins which adsorbed to a DEAE cellulose resin were eluted with a linear gradient of 0-300 mM NaCl resulting in a further 3.5-fold purification. The final purification step involved affinity chromatography. This column consisted of GTP coupled to an acyl hydrazide derivative of agarose. Guanosine triphosphate is a substrate of PEPCK and the enzyme binds to this column-bound ligand in the presence of manganese (86). Purified PEPCK was then eluted from the column with a 1 mM GTP solution. Affinity purification resulted in an additional 3-fold purification of the enzyme (Table 2). Based on total activity, 10% of the enzyme was recovered in a highly purified form. The final specific activity was 16 U/mg which is comparable to that

Table 2: Purification of PEPCK.

	Total Protein mg/ml	Total Activity U/ml	Specific Activity[a] U/mg	% Yield
Mouse liver cytosol	641	252	0.39	100
Ammonium sulfate precipitate	180	205	1.1	81
BioGel P6 gel filtration column	116	188	1.6	75
DEAE ion exchange column	16.8	98	5.8	39
GTP-hexane-agarose affinity column	1.5	24	16.0	10

[a] Defined as nanomoles $^{14}CO_2$ fixed per minute per mg protein.

used by Ballard and Hanson (5).

In order to determine enzyme purity, the affinity column eluate was analysed by SDS-PAGE. There is a distinct and single band (Figure 11). Its molecular weight was calculated by the method of Weber and Osborn (165) to be 71,500 daltons. This is in good agreement with the value of 70,600 daltons reported by Iynedjian et al (86). The purified enzyme was used to immunize a New Zealand white rabbit in order to obtain anti-PEPCK antibody. As shown in Figure 12, an antibody response was elicited against the purified enzyme preparation as the immune serum precipitated a single protein from a high-speed (100,000 x g), 15% (w/v), mouse liver supernatant fraction.

In order to further show that a unique protein reacted with the immune serum, the methods of immunoelectrophoresis and Western blotting were employed. The presence of two distinct bands that differ in electrophoretic mobility are shown in Figure 13. The presence of two distinct proteins was confirmed by the observation of a doublet band upon autoradiography of a Western blot (Figure 14). The immune serum was derived following immunization with purified PEPCK; therefore, the reason for the presence of two distinct antigens is unclear. It is possible that during the preparation of the liver supernatants some mitochondria may have been ruptured and leakage of mitochondrial PEPCK (mPEPCK) occurred. This isoenzyme is immunologically distinct from cytosolic

Figure 11: SDS-polyacrylamide gel electrophoresis of purified phosphoenolpyruvate carboxykinase (PEPCK). Details of the purification scheme are detailed in the Materials and Methods section. Lane a = molecular weight standards: thyroglobin (half unit), 330K; ferritin (half unit), 220K; lactate dehydrogenase, 140K; phosphorylase b, 94K; albumin, 67K; catalase, 60K; ovalbumin, 43K; lactate dehydrogenase (subunit), 36K; carbonic anhydrase, 30K; trypsin inhibitor, 20.1K; and lactalbumin, 14.4K. Lane b = high-speed (100,000 x g) mouse liver supernatant, 15% (w/v). Lane c = purified PEPCK.

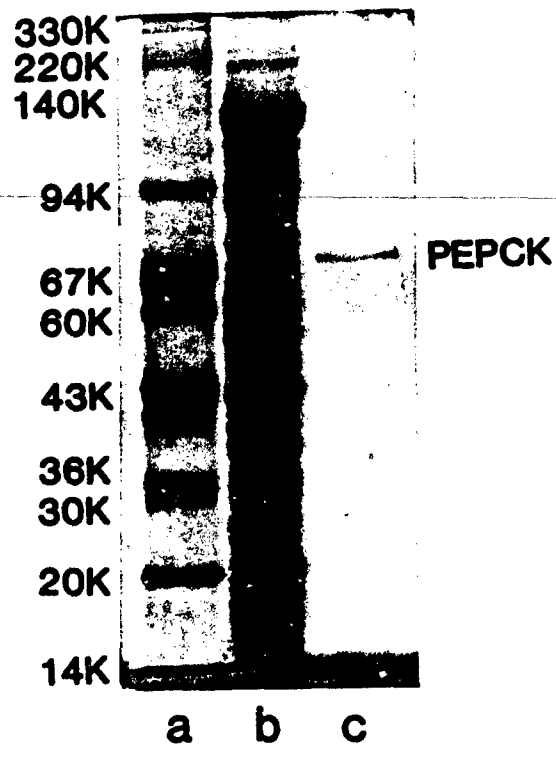

330K
220K
140K
94K
67K
60K
43K
36K
30K
20K
14K
PEPCK
a
b
c

Figure 12. Immunodiffusion pattern showing an immunoprecipitate. Immune serum, obtained from a New Zealand white rabbit after immunization with purified PEPCK, is diluted from 1:1 to 1:128, counterclockwise, in the outer wells. A high-speed (100,000 x g) mouse liver supernatant, 15% (w/v), is in the center well.

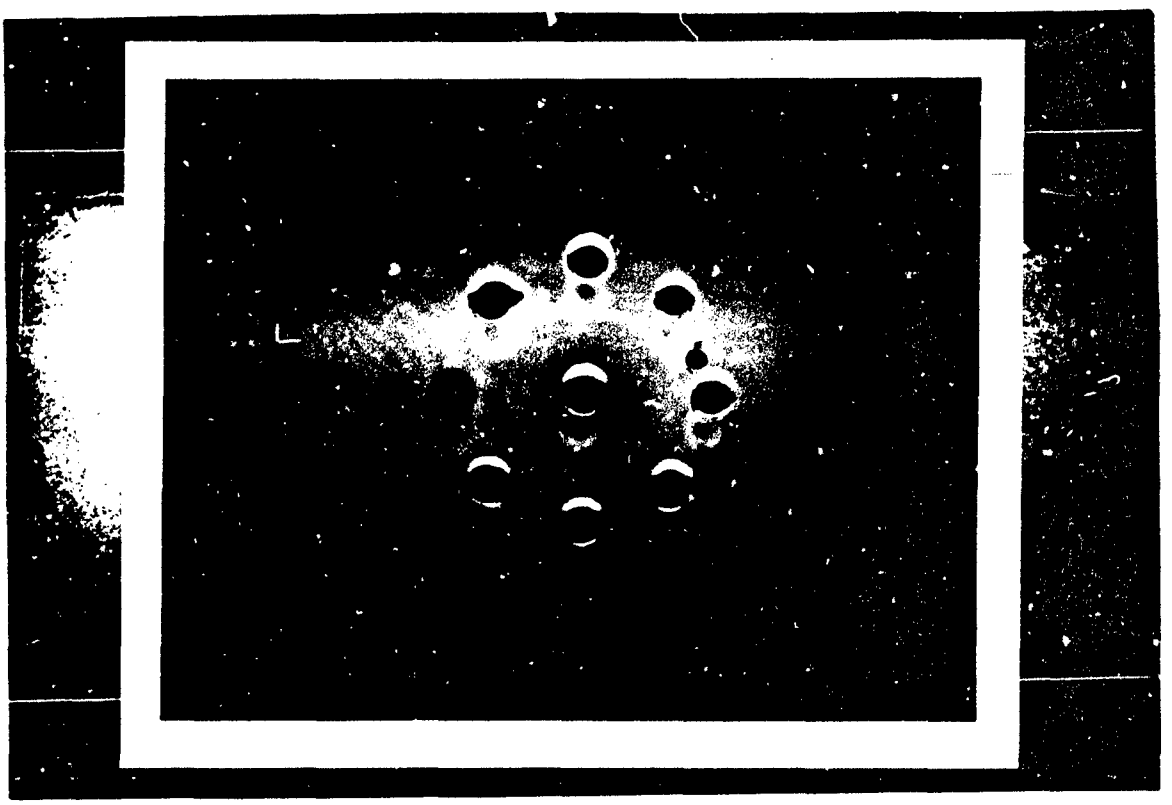
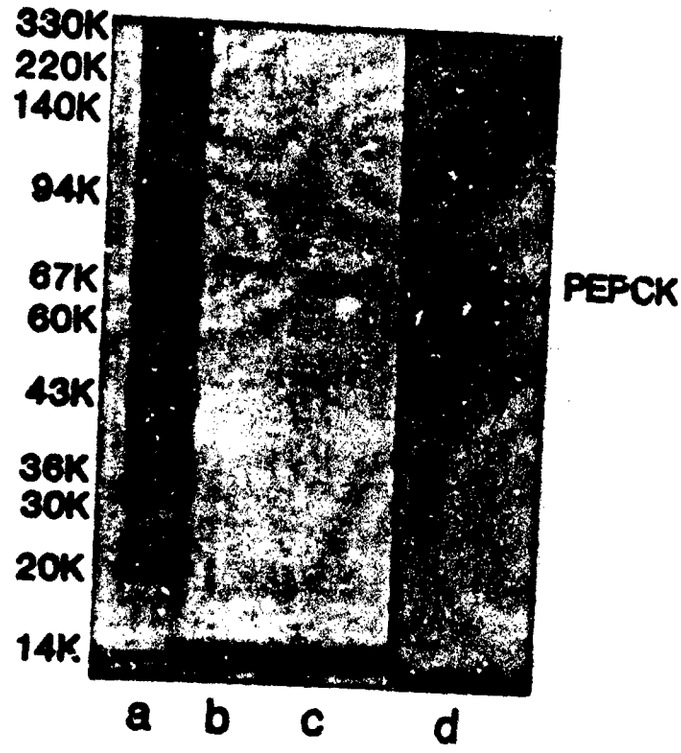


Figure 13: Immunoelectrophoresis of a high-speed (100,000 x g) mouse liver supernatant, 15% (w/v), for 90 minutes at 3 mA. An immunoprecipitate was observed 24 hours after incubation of the electrophoresed antigen with rabbit anti-mouse PEPCK placed in the center trough.

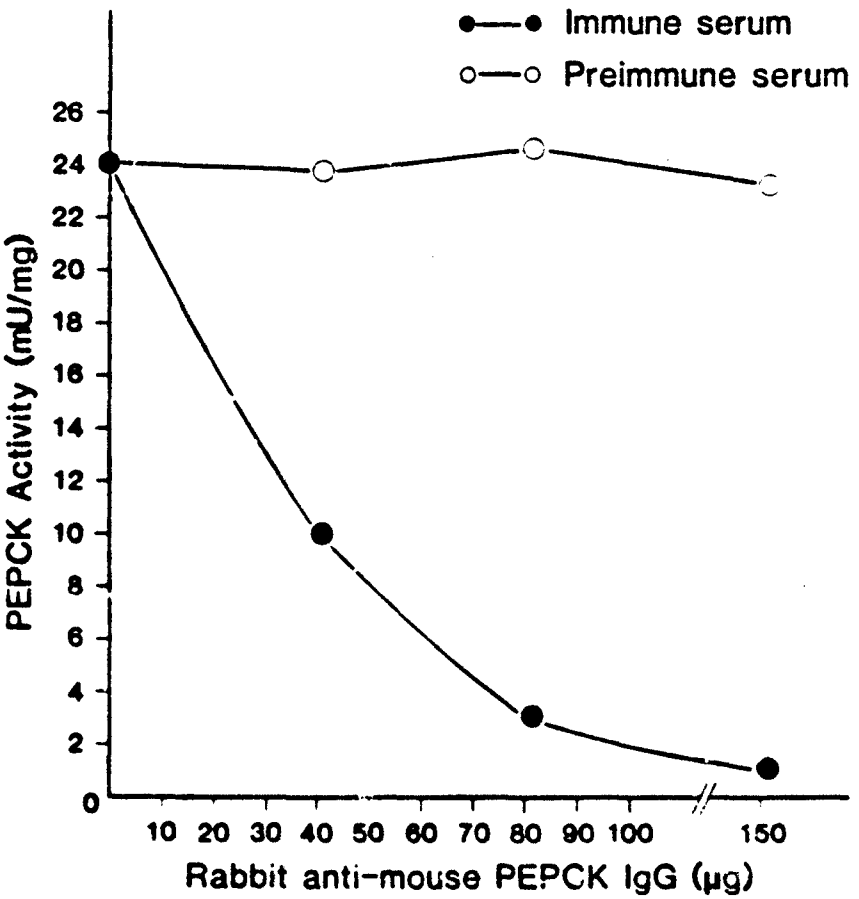
Figure 14: Western blot following SDS-PAGE. Details are provided in the Materials and Methods section. Lanes a-c represent one-half of the SDS-PAGE gel which was stained by conventional methods. Lane d represents the other half of the SDS-PAGE gel which was transferred to nitrocellulose and incubated with antiPEPCK antibody and ^{125}I-labelled protein A. Lane a = molecular weight standards (refer to figure 13 for details). Lane b = 40 ul of purified PEPCK. Lane c = 20 ul of purified PEPCK. Lane d = high-speed (100, 000 x g) mouse liver supernatant, 15% (w/v), blotted onto nitrocellulose.


330K
220K
140K
94K
67K
60K
43K
36K
30K
20K
14K
PEPCK
a
b
c
d

PEPCK (cPEPCK) (86). A small amount of mPEPCK may have contaminated the purified enzyme used in the present study and although undetectable by the conventional stain employed in SDS-PAGE, it may have elicited an immune response. Therefore, the two proteins may represent the presence of both anti-mPEPCK and anti-cPEPCK antibodies in the immune serum. It is also possible that, although immunologically distinct, residual amounts of mPEPCK in the liver due to leaky mitochondria may cross-reacts with anti-cPEPCK in the immune serum. However, based upon the intensity of both bands in the Western blot, this appears unlikely, unless mPEPCK is more antigenic than cPEPCK. An alternative explanation is that anti-PEPCK cross-reacts with a liver protein, other than PEPCK, which exhibits similar immunoelectrophoretic mobility and comparable molecular weight to that of PEPCK (Figures 13 and 14).

To further clarify the specificity of the immune serum against PEPCK, a PEPCK-antiPEPCK immune complex was precipitated with protein A of Staphylococcus aureus. Following immunoprecipitation the supernatant was assayed for the presence of PEPCK. The absence of activity with increasing concentrations of antibody verify the specificity of the immune serum (Figure 15). Addition of preimmune serum, in the presence of protein A, had no effect on decreasing the activity level of PEPCK; therefore, nonspecific immunoprecipitation was not a factor in the assay.

Figure 15: Titration of rabbit anti-mouse PEPCK IgG. A fixed amount or PEPCK (24 mU/mg) in a high-speed (100,000 x g) mouse liver supernatant was incubated with anti-PEPCK. the immune complex was then precipitated with protein A, S. aureus, Cowan I strain. Following centrifugation, the supernatant from immune serum (•---•) and preimmune serum (o---o) incubations were assayed for PEPCK activity.


Immune serum
Preimmune serum
PEPCK Activity (mU/mg)
0
2
4
6
8
10
12
14
16
18
20
22
24
26
10 20 30 40 50 60 70 80 90 100 150
Rabbit anti-mouse PEPCK IgG (µg)

MEASUREMENT OF AMOUNT OF MOUSE HEPATIC PEPCK BY ENZYME LINKED IMMUNOSORBANT ASSAY (ELISA) AND COMPARISON WITH ACTIVITY LEVELS MEASURED BY CARBOXYLATION WITH RADIO-LABELED BICARBONATE: Hepatic PEPCK is readily induced by hydrocortisone (126,158), cAMP (169,170), and fasting (59, 148). LPS and GAF are known to inhibit the induction of PEPCK by hydrocortisone (18,130) and fasting (131), but not induction by cAMP (66). Inhibition of this key gluconeogenic enzyme may be directly related to the animals increased sensitivity to stress. In the above studies, quantitation of PEPCK is measured by activity, defined as nanomoles of $^{14}CO_2$ fixed per minute. Only one study has attempted to directly quantitate the amount of enzyme and this was performed using the method of radial immunodiffusion (91). The purpose of this experiment was to design an enzyme linked immunosorbant assay (ELISA) for direct quantitation of hepatic PEPCK and compare the OD readings obtained by ELISA with the activity levels of the enzyme. Rabbit anti-mouse PEPCK IgG was obtained by immunizing a New Zealand white rabbit with purified PEPCK and isolating the IgG fraction (complete details are given in the Materials and Methods section). the specificity of this anti-PEPCK antibody was illustrated previously (Figure 15).

Hepatic PEPCK from the liver supernatant of normal mice had an OD 490 of 0.093 by ELISA (Table 3). An OD of 0.084 was seen when mice were treated with 25 ug of LPS 4

Table 3: Catalytic activity vs ELISA antigenicity of hepatic PEPCK. Numbers in parenthesis represent the number of mice in that group.

[a] Defined as nanomoles of $^{14}CO_2$ fixed per minute per mg protein.

[b] Defined as OD 490 of oxidized OPD after 30 minute reaction time with HRP and H_2O_2.

[c] Ratio of specific activity to OD 490 (ELISA).

[d] Normal, uninduced mice.

[e] 25 ug LPS given (iv) 5 hours prior to hepatectomy.

[f] 50 mg/kg Hydrocortisone given (sc) 4 hours prior to hepatectomy.

[g] 24 Hour fast prior to hepatectomy.

[h] 25 ug LPS given (iv) 1 hour prior to 50 mg/kg hydrocortisone (sc) and 5 hours prior to hepatectomy.

[i] Serum collected 6 days after RES activation with 700 ug C. parvum. Given (0.4 ml,iv) concurrent with hydrocortisone.

[j] Serum collected 2 hours after challenge with 25 ug LPS. Given (0.4ml,iv) concurrent with hydrocortisone.

[k] Serum collected 2 hours after challenge with 25 ug LPS in mice primed 6 days earlier with C. parvum. Given (0.4 ml, iv) concurrent with hydrocortisone (sc).

Table 3:

Treatment	Specific Activity[a] mU/mg	ELISA[b] OD 490	Specific Activity[c] ELISA
Uninduced[d]	19.9 1.0 (17)	.093 .002 (17)	214
LPS[e]	20.0 1.1 (14)	.084 .004 (14)	238
Hydrocortisone[f]	42.4 1.8 (17)	.154 .004 (17)	275
Fasted[g]	45.0 3.6 (12)	.168 .005 (12)	268
Hydrocortisone:			
LPS[h]	18.3 1.1 (5)	.090 .002 (5)	203
Cps[i]	36.2 2.2 (11)	.139 .005 (11)	260
ES[j]	36.0 3.0 (11)	.137 .005 (11)	262
CpES[k]	22.4 2.8 (8)	.094 .007 (8)	238

hours prior to hepatectomy. Thus, normal and LPS-treated mice have essentially the same levels of hepatic PEPCK ($P > 60$). In addition, the specific activity of PEPCK did not differ significantly between normal mice and mice pre-treated with LPS, 19.9 mU/mg and 20.0 mU/mg, respectively (Table 3). An increase in both the activity and the amount of PEPCk was shown upon treatment with a dose of 50 mg/kg of hydrocortisone given 4 hours prior to liver extraction. An OD of 0.154 was measured by ELISA in this group. This is a significant increase in the amount of enzyme when compared to normal mice ($P < 0.05$). Likewise, the specific activity of PEPCK was substantially elevated by hydrocortisone from a baseline level of 19.9 mU/mg to 42.4 mU/mg. Table 3 also shows the effects of a 24 hour fast on hepatic PEPCK. These mice exhibited the greatest rise in the amount of enzyme and corresponding activity, 0.168 OD by ELISA and 45.0 mU/mg by carboxylation, respectively. Although this increase is significant with respect to normal levels of PEPCK ($P < 0.05$) it is only slightly higher than that obtained by hydrocortisone ($P = 58$).

To show that the induction of PEPCK by hydrocortisone was inhibited by a serum factor, mice made tolerant to LPS were treated with 50 mg/kg of hydrocortisone that was given concurrently with serum rich in GAF (CpES). CpES completely blocked the induction of PEPCK by hydrocortisone (Table 3). As measured by ELISA the OD was 0.094 for the group

treated with hydrocortisone plus CpES and 0.093 OD for the normal group (P = 96). The activity levels of PEPCK were also similar for these two treatment groups, 19.9 mU/mg for normal mice and 22.4 mU/mg for mice treated with hydrocortisone and CpES (P = 52). Thus, there is no significant difference between measurable PEPCK in normal mice and in mice given hydrocortisone concurrent with CpES. Control serum was also tested to show that inhibition of PEPCK by hydrocortisone was due neither to residual LPS nor to some nonspecific inhibitor in the serum. Endotoxin serum (ES) collected 2 hours after challenge with 25 ug of LPS and C. parvum serum (CpS) collected from mice receiving C. parvum 6 days earlier did not significantly alter the induction of PEPCK by hydrocortisone. Measurements by ELISA show that mice given CpS or ES plus hydrocortisone had PEPCK levels corresponding to 0.139 and 0.137 OD readings, respectively (Table 3). These results were slightly lower than those for hydrocortisone induction. This difference, though statistically insignificant (P < 0.05), appears to be consistent. The activity levels were also lower than results obtained by hydrocortisone alone (42.4 mU/mg). The activity of PEPCK was 36.2 mU/mg in mice treated with hydrocortisone plus CpS and 36.0 mU/mg in mice treated with hydrocortisone plus ES. The reason for the slight inhibition of induction of PEPCK by ES and CpS may be due to the presence of slightly elevated amounts of GAF (65).

A linear relationship between carboxylation activity

levels and amount of enzyme (ELISA) can be established by comparing the ratio of activity to the amount of enzyme (Table 3). This is statistically significant as calculated by the Spearman rank correlation coefficient ($r = 0.93$). Therefore, induction of PEPCK by hydrocortisone or by fasting resulted in enhanced levels of activity of the enzyme and this correlates directly with increased amounts of the enzyme as measured by ELISA.

DISCUSSION

The antagonism between glucocorticoids and endotoxin has been known for more than 30 years (49). Although much research has been conducted during this time, only recently has the basis for this conflict between these hormones and LPS been attributed to a serum factor produced by cells of the RES (21,111,114,115). Since an adrenal cortical response is necessary for survival of animals that are or have been subjected to stress (140), the nature of how LPS antagonizes the protection afforded by the adrenals was elucidated by subjecting mice to stressful environments. It should be noted that both glucocorticoids and mineralcorticoids are released during the stress-induced cortical response, but the role played by mineralcorticoids is believed to be of little importance to survival (6). The discussion that follows will focus, therefore, on the role of glucocorticoids.

Surgically adrenalectomized animals are extremely sensitive to the lethal effects of LPS (33,37,128), the stress of cold or heat (74), trauma (142), and other types of stress. Accordingly, glucocorticoids are necessary for an animal's survival under these conditions. When the glucocorticoid response is blocked by LPS, the deliterious effects of stress are magnified in the endotoxinemic animal. Previte and Berry (125) showed that mice housed at 5°C are killed, while mice housed at 25°C survive infection with

Salmonella typhimurium strain Sr-11-A. This observation was extended to purified endotoxin when Berry (15) showed that the LD_{50} for LPS was dramatically reduced in mice exposed to environmental temperatures of 5°C or 37°C. Couch et al (43) also showed that mice exposed to 5°C are sensitized to lethality due to LPS. This present study confirm these early reports. In addition, it was found that mice given a small dose of LPS, detoxified by irradiation with 150 kiloGrays from a ^{60}Co source (RD-LPS) at the beginning of cold exposure also led to poor survival. Mice were sensitized 700-fold to LPS and 40-fold to RD-LPS after 10 hours of cold exposure (Figure 1). At 37°C, lethality was even more pronounced. At 10 hours, a 10,000-fold and a 2,000-fold sensitivity was seen for LPS and RD-LPS, respectively (Figure 2).

The reason for enhanced sensitivity to LPS and RD-LPS at 37°C is not clear. Heroux and Hart (74) reported that the amount of adrenal cortical extract required by adrenalectomized rats was the same at 5°C and at 37°C. Therefore, the greater sensitivity of mice to LPS or RD-LPS at 37°C, relative to 5°C, cannot be explained on the basis of a difference in demand for glucocorticoids. A hyperthermic response of only 2-3°C has been measured at about the time the animals die when housed at 37°C (Figure 4). By comparison, a decrease in body temperature, by as much as 18°C, occurs at the time of death when mice are housed at 5°C. Thus the degree of cooling is much greater than the degree of a rise in body

temperature. This suggests that regulation of hyperthermia may be more critical than regulation of hypothermia, at least in terms of the number of degrees regulated. This difference in response during exposure to heat or cold may account for the greater sensitivity to heat.

Another difference in how animals respond to heat and cold may involve the release of B-endorphins. These endogenous opiates appear to play a role in adaptation to heat but not to cold. Holaday et al (82) showed that rats exposed to heat exhibit a greater hyperthermic response, by about 0.5°C, when treated with the opiate antagonist naloxone. This small difference in body temperature was associated with an increase in weight loss and an increase in the number of escape attempts (82). Goldstein (64) did not observe a significant difference in the hypothermic response of rats during exposure to cold in the presence or absence of naloxone. The results presented in this thesis also show that naloxone does not alter the hypothermic response of mice during exposure to cold (Figure 3), in agreement with the work of Goldstein (64). However, this work does not confirm the conclusion made by Holaday (82) since treatment with naloxone did not result in increased hyperthermia in mice kept at 37°C (Figure 4). One obvious difference between these studies and those of Holaday is the fact that in this work animals were poisoned with LPS. Since LPS induces the release of B-endorphins (81), the dose level of naloxone which was administered may not

have been sufficient to antagonize the increased level of B-endorphin. The dose of naloxone used was 5 mg/kg which is 50 times greater than that used by Faden et al (52,53) to reverse LPS-induced hypotensive shock, but is only one-half the dose used by Holaday (82). We also measured colonic temperature over a period of hours whereas Holaday measured colonic temperature over a period of minutes. These obvious differences in experimental design could account for the different results. In addition, naloxone failed to alter the mortality rate of mice that were poisoned with LPS and then housed at either 5°C or 37°C (Figures 3 and 4). This observation is important since it negates several conclusions which suggest that B-endorphins may serve as lethal factors. First, the levels of mRNA for the B-endorphin and adrenocorticotropin hormone (ACTH) precursor, proopiomelanocortin (POMC), are increased following adrenalectomy (24). Also, adrenalectomized rats have elevated levels of B-endorphins in their blood (1) and these animals are sensitized to stress (33,37,74,142). Exogenous dexamethasone, acting as a feedback inhibitor of POMC (48) prevents hyperendorphinemia in adrenalectomized rats (24) and also affords protection against stress (Figures 3,4 and 9). Finally, B-endorphins are produced in response to LPS and they have been implicated in hypotensive shock (52,81,98). Increased levels of these opiates can thus be correlated directly with a decrease in the survival of animals unable to elicit an adrenal cortical

response during stress. It can be postulated that glucocorticoids, endogenous or exogenous, may act by decreasing the level of B-endorphin and hence, increase survival. However, this study does not show increased survival of endotoxinemic mice during cold or heat stress when pretreated with the endorphin antagonist naloxone (Figure 3 and 4). The role of these opiates remains enigmatic in unstressed mice as well. Treatment with naloxone results in increased survival of LPS-poisoned dogs (52) and monkeys (83), but not in rats (81), piglets (145) or Yucatan minipigs (57).

The lethal effects of LPS and RD-LPS were also studied during stress conditions other than temperature stress. When mice are primed with a subcutaneous dose of LPS or RD-LPS they become sensitive to a second, normally sub-lethal dose, of LPS 6 hours later (Figure 8). Since serum rich in GAF sensitizes mice to the lethal effects of LPS (43), it is probable that the priming dose of LPS or RD-LPS may cause the release of GAF from cells of the RES and subsequent sensitization to a later dose of LPS. Figure 9 also shows that when hydrocortisone is given concurrently with the priming dose of LPS, mortality rates, due to the second dose of LPS, are reduced. This indicates that hydrocortisone inhibits the release of GAF by the priming dose, although antagonism of GAF at some other metabolic level cannot be excluded. When hydrocortisone is given 6 hours after the priming dose and concurrently with the second dose of LPS

the protection afforded is considerably less (Figure 9). This implies that the anti-glucocorticoid effect of GAF is more intense when it is released prior to exposure to hydrocortisone. Thus the antagonism between endotoxin and glucocorticoids appears to be bidirectional and the early effects of one may block the later effects of the other. This may be a critical factor during stress where functional adrenalectomy is fatal. Support for the pharmacological action of GAF was unknowingly introduced by Geller (61) in an earlier study. He observed that both adrenalectomized mice and mice whose RES had been stimulated by zymosan were sensitized to the same extent to LPS. In agreement with the work of Geller Table 1 shows that mice whose RES is activated with heat-killed Corynebacterium parvum are sensitized to the lethal effects of LPS and Rd-LPS. Since elevated levels of GAF are released following treatment with zymosan and LPS (65), it is likely that a decrease in survival is due to functional adrenalectomy by GAF. This is comparable to the sensitization of surgically adrenalectomized mice to the lethal effects of endotoxin.

Another type of stress observed during this study was that of trauma, introduced by tourniquet shock. Mice whose hind limbs were subjected to tourniquet application for 1 hour were sensitized to the lethal effects of LPS by more than 300-fold (Figure 10). At 24 hours after trauma, the LD_{50} for LPS was 1.3 ug. Deaths were mediated by the effects of

endotoxin as the tourniquet application alone was nonlethal in unpoisoned mice (Figure 10). Since adrenalectomized animals are also sensitized to tourniquet shock (142), it seems reasonable to suggest that LPS has, through the release of GAF, sensitized the mice to this trauma by antagonizing the adrenals. This suggestion is reinforced by the observation that lethality could be attenuated by exogenous hydrocortisone (Figure 10).

A direct analogy between surgical adrenalectomy and adrenal antagonism by LPS or RD-LPS can be ascertained by the findings that treatment with either results in poor survival of animals subjected to stress. However the degree of adrenal suppression is not the same since LPS is more lethal than RD-LPS during exposure to cold or heat (Figures 1 and 2). Radiodetoxification of LPS results in decreased levels of ketodeoxyoctonate (KDO) groups and altered glycosamine and fatty acid groups (23) generating a more hydrophobic molecule this may alter the time course of the interaction of RD-LPS with cells of the RES. Although macrophages are believed to be the cells responsible for the production of LPS-induced mediators (19,20), the interaction between these cells and LPS is ill-defined and further research in this area may provide some insight into the decreased, or residual toxicity seen with RD-LPS. Nonetheless. this data shows that RD-LPS is a potent toxin in mice during stress (Figures 1,2 and 9) and this lethality, as well as lethality

due to toxic LPS under similar conditions, is mediated by a serum factor which may act to functionally adrenalectomize the animal.

It is known that serum collected 2 hours after challenge with LPS in mice whose RES has been activated contains elevated levels of glucocorticoid antagonizing activity (111,112,114). The responsible factor was referred to as glucocorticoid antagonizing factor (GAF) based on its ability to inhibit the induction of PEPCK by hydrocortisone (111, 114). GAF has been partially characterized as a protein with a molecular weight of approximately 150,000 daltons and it is sensitive to both heat and trypsin (114). This study shows that GAF-rich serum can be obtained when either LPS of RD-LPS is used as the inducing agent in mice whose RES has been primed with heat-killed Corynebacterium parvum. This serum was designated CpES for C. parvum-LPS treatment and CpdES for C. parvum-RD-LPS treatment. As shown in Figures 5 and 6, both CpES and CpdES are highly toxic for mice during exposure to cold or heat. In addition, this serum also sensitizes mice to an otherwise nonlethal traumatic shock (Figure 10). All mice treated with this serum were made tolerant to the effects of LPS, it can therefore be concluded that lethality was indeed due to a humoral factor, presumably GAF, and not due to LPS. It becomes apparent that serum collected after proliferation of cells of the RES followed by exposure of these cells to the toxin, results in a yield of GAF which is

approximately the same whether RD-LPS or LPS is injected. The relative significance of these findings remains to be clarified. When RD-LPS was used as a stimulant for inducing nonspecific resistance (NSR) without hypotensive effects associated with the toxic preparation, Bértók (23) showed that the administration of RD-LPS, given at least 48 hours prior to challenge resulted in the protection of dogs against hemorrhagic shock and protected rats against tourniquet or septic shock. In contrast, these studies showed RD-LPS to be lethal when given at the time stress is initiated (Figures 1,2 and 9). This lethality was also shown to be mediated by a serum factor (GAF) produced in response to RD-LPS (Figures 3 and 4). It is likely that the deliterious effects of GAF occur early. In support of this, Moore et al (115) showed that the maximal level of GAF is obtained 2 hours after challenge with LPS and this level rapidly subsides to normal levels after about 3 hours. Consequently, by the time Bértók applied stressor stimuli 48 hours after challenge with RD-LPS the biological effects of GAF may have disappeared. Therefore, the time between challenge with RD-LPS and the induction of stress is crucial for the sensitization of the animal to, or the protection of the animal against, stress. Since mice are sensitized to the antagonism of their adrenal response by GAF, it seemed logical to study the effect of exogenous glucocorticoids in protecting these mice.

Glucocorticoids are known to attenuate lethality

when LPS is given to mice housed at 25°C (12,14,61,90,91, 109), at 5°C (125) and at 37°C (15). This work has shown that exogenous hydrocortisone results in increasd survival of mice poisoned with LPS at 5°C (Figure 3) and 37°C (Figure 4). Hydrocortisone was also shown to protect against a sub-lethal dose of LPS given to mice which had been primed earlier with a small dose of LPS or RD-LPS (Figure 9) and against traumatic shock (Figure 10). Thus glucocorticoids are efficacious in protecting mice during a variety of conditions where the animal is poisoned with LPS and also subjected to various conditions of stress. This exemplifies the importance of glucocortcoids and their potential antagonism of LPS. In order for these exogenous glucocorticoids to afford protection, they must be given no later than 1 hour after challenge with LPS (14). This is most likely due to immunosuppression of macrophages (47) with diminished release of serum mediators, such as GAF. Once these humoral factors have been released and are allowed to exert their biological effects, treatment with hydrocortisone is ineffective. This was shown where hydrocortisone given 2 hours prior to CpES afforded protection (Figures 7 and 8) while hydrocortisone given concurrently with CpES did not protect (Figure 10). In addition to immunosuppression, glucocorticoids may afford protection by other means.

Another mechanism for the action of glucocorticoids may be due to maintenance of carbohydrate homeostasis. Wolfe

et al (171) showed that the hypotensive effects of LPS in dogs was not severe enough to account for their deaths. However, the hypoglycemic state manifested by these animals was incompatible with life. In addition, all dogs which survived poisoning with LPS were able to maintain their blood glucose levels. Menten and Manning (108) were the first to observe total depletion of carbohydrates in rabbits poisoned with LPS. This was later shown to be due to a combination of factors, including depletion of hepatic glycogen stores (10,13,58,94) and hyperinsulinemia (173). In order to maintain a sufficient caloric balance the animal must rely on increased consumption of exogenous carbohydrates or an increase in the rate of gluconeogenesis. Both of these parameters are blocked by LPS. Exogenous carbohydrate intake is blocked because LPS severely limits the consumption of food and water by inhibiting gastric emptying (159). Also, LPS blocks the synthesis of glucose from non-carbohydrate sources (11,102,147) even though protein catabolism is unaltered (11). Thus caloric insufficiency may be a critical factor in the demise of endotoxinemic animals. It is interesting to note that the LPS-induced glycogen depletion in rats is abolished by adrenalectomy (139), yet adrenalectomized animals are still sensitized to cold (74). The most likely explanation for this is that glycogenolysis is regulated by the cholinergic response from the adrenals (139) and adrenalectomized animals in the cold may have

sufficient glycogen stores, but these are unobtainable and thus of little value in caloric conservation.

Endotoxinemic mice treated with hydrocortisone and exposed to cold or heat survive longer and are able to regulate their body temperature more efficiently than mice treated with LPS alone (Figures 3 and 4). In a previous study Previte and Berry (125) also showed the ability of cortisone to protect mice poisoned with LPS during exposure to cold. This protection may be due to the maintenance of carbohydrate reserves and preservation of energy as hydrocortisone induces protein catabolism (100) and gluconeogenesis (50). The importance of glucocorticoids during exposure to cold is illustrated by the findings that mice housed at 5°C demonstrate increased levels of endogenous glucocorticoids (92) and PEPCK (43), the rate-limiting enzyme in gluconeogenesis. This enables sequestration of carbohydrate reserves in order to cope with the additional energy requirements during stress to cold, and possibly other types of stress. Kun (94) showed that rats poisoned with LPS are unable to convert glucose or pyruvate into hepatic glycogen. In addition, Berry (13) observed that hepatic glycogen dropped significantly below normal levels in the presence or absence of cortisone. The situation is compounded further by the finding that LPS inhibits gluconeogenesis (10,102,147). This is due to the inhibition of PEPCK by GAF, as Couch et al (43) showed that endotoxin tolerant mice housed at 5 C retain fully inducible

levels of PEPCK when given LPS but that addition of serum rich in GAF completely blocked this induction and hypoglycemia ensues. This confirms the work of others who have shown the ability of GAF to block induction of PEPCK by hydrocortisone (65,111,112,114). Carbohydrate homeostasis may thus be a crucial factor for the survival of the endotoxinemic animal, especially during stress where enhanced sensitization to GAF is observed.

The question remains as to whether LPS sensitizes the animal to stress or whether the stressed animal is sensitized to LPS. This is made difficult by the fact that adrenalectomized animals are sensitized to both stress (74, 142) and LPS (33,37). This study shows that the lethal effect of LPS during stress is mediated by a serum factor whose effects can be reduced by prior treatment of hydrocortisone (Figures 7 and 8) but not by hydrocortisone given concurrently with serum that contains the serum factor GAF (Figure 10). The anti-glucocorticoid activity of GAF is demonstrated by its ability to inhibit the induction of hepatic PEPCK by hydrocortisone (Table 3). The ability of GAF to suppress the animals adrenal response suggests that LPS or RD-LPS may sensitize the animal to stress, where an adrenal cortical response is obligatory for survival (140). Most of the preceeding work has dealt with the biological aspects of the antagonism of glucocorticoids by endotoxin, specifically the LPS-induced serum factor GAF. Experiments of this type

are often difficult to assess due not only to differences in response of animals of different genera, but to individual differences in response of animals in the same genus as well. Environmental conditions, general handling, and overall care may also be important to the animal's biological response. Thus the mechanism by which GAF exerts adrenal steroid antagonism must be resolved not only in terms of death or survival of animals, but also at the cellular level.

In order to understand how GAF antagonizes glucocorticoids, it is essential to understand how these hormones produce their effects. Glucocorticoids enter target cells and bind noncavalently and reversibly to cytosolic receptor proteins (138). Following dephosphorylation, these receptor proteins associate with nuclear chromatin to initiate transcription (138). Higgins and Gehring (75) showed this to be site specific. Thus it would follow that antagonism by GAF may also be site specific. In support of this, it has been shown that GAF blocks the glucocorticoid induction of PEPCK but not tyrosine aminotransferase (TAT) in mouse peritoneal exudate cell cultures (65). By using [^{3}H]dexamethasone as a stimulant, Berry and Shackleford (22) showed that LPS neither altered the entry of the hormone into hepatic cells nor inhibited the binding of the hormone to cytosolic receptor proteins. Adrenalectomized mice were used to limit the effect of endogenous glucocorticoids. This suggests that the hormone exerts its effects at a transcriptional or post-

transciptional level. It has been shown that induction of PEPCK by fasting (131) or hydrocortisone (Table 3) is the result of an increase in amount of enzyme and not altered enzyme activity. Rippe and Berry (131) also showed by radial immunodiffusion that the amount of PEPCK, induced by fasting, could be blocked by LPS. Employing the method of ELISA, this study shows that hydrocortisone induces an increase in the amount of PEPCK and this could be inhibited with either LPS or the serum rich in GAF (CpES) (Table 3). This is due to inhibition of synthesis and not a result of modification of the enzyme resulting in dysfunctional enzyme activity as the amount of PEPCK measured by ELISA correlated directly with the activity of the enzyme ($r = 0.93$ by the Spearman rank correlation coefficient shown by the activity to ELISA ratio in Table 3). Induction of PEPCK requires active protein synthesis as actinomycin D inhibits the increase in the amount of enzyme (131). In addition de novo synthesis of PEPCK is probable since the amount of mRNA for PEPCK is increased following induction by glucocorticoids (2,8,38,39,85,107,164). The fact that levels of mRNA for PEPCK are not induced by hydrocortisone during endotoxinemia (22) strongly suggest that antagonism by GAF occurs at the level of transcription. Further studies involving gene expression of readily available DNA have provided additional information in this area of research.

The transcription of mammary tumor virus (MTV) can

be stimulated 100-fold by glucocorticoids (129). A 1450 base pair fragment of viral DNA containing a long terminal repeat (LTR) is necessary for this induction to occur (54). This is highly suggestive of a direct interaction of glucocorticoids with DNA at the level of transcription. Also, binding of the promoter region appears to be the rate-limiting step for induction of transcription of MTV DNA by glucocorticoids as the rate of initiation of transcription was unaltered (160). This same study showed that post-transcriptional events, splicing and polyadenylation, were also unaffected. It does not seem unreasonable to propose the same mode of action by glucocorticoids acts in the regulation of PEPCK, as well as other target enzymes. If glucocorticoids act at site specific promoter regions of target DNA, the antagonism by GAF may also occur at this level. Several possibilities need to be examined. GAF may alter the dephosphorylation of the cytosolic receptor protein, inhibit the transport of the activated receptor protein across the nuclear membrane, or block the binding of the phosphoprotein to site specific DNA sequences. Isolation and purification of GAF should lead to more meaningful experiments on the mechanism by which it antagonizes glucocorticoids.

These studies clearly show that a serum borne mediator (GAF), produced by cells of the RES in response to LPS or RD-LPS, antagonizes the effects of hydrocortisone in inducing levels of hepatic PEPCK and subsequently gluco-

neogenesis. This is associated with a severe and terminal hypoglycemia which may be incompatible with life. Animals placed under conditions of stress where an adrenal cortical response is required for survival are highly sensitized to the lethal effects of LPS or RD-LPS and their respective GAF-rich sera (CpES and CpdES, respectivley). Purification of GAF would enable further research into the specific physiological and molecular derangements incurred by this glucocorticoid antagonist.

BIBLIOGRAPHY

1. Akil, H., S.F. Watson, J.D. Barches, C.H. Li. 1979. B-endorphin immunoreactivity in rat and human blood: radioimmuncassay, comparative levels and physiological alterations. Life Sciences 24: 1659-1665.

2. Andreone, T.L., E.G. Beale, R.S. Bar , and D.K. Granner. 1982. Insulin decreases phosphoenolpyruvate carboxykinase (GTP) mRNA activity by a receptor mediated process. J. Biol. Chem. 257: 35-38.

3. Arinze, I.J., A.J. Garber and R.W. Hanson. 1973. The regulation of gluconeogenesis in mammalian liver. J. Biol. Chem. 248: 2266-2274.

4. Atchley, D.W. 1930. medical shock. J.A.M.A. 95: 385-389.

5. Ballard, F.J. and R.W. Hanson. 1969. Purification of phosphoenolpyruvate carboxykinase from cytosol fraction of rat liver and the immunochemical demonstration of difference between this enzyme and the mitochondrial phosphoenolpyruvate carboxykinase. J. Biol. Chem. 244: 5625-5630.

6. Baxter, J.D. and G.G. Rousseau. 1979. Glucocorticoid hormone action: An overview. In Glucocorticoid Hormone Action (J.D. Baxter and G.G. Rousseau, eds.), Springer-Verlog, New York, pp. 1-24.

7. Beale, E.G., C.S. Katzen and D.K. Granner. 1981. Regulation of rat liver phosphoenolpyruvate carboxykinase (GTP) messenger ribonucleic acid activity by N^6,O^2-dibutyryladenosine 3',5'-phosphate. Biochem. 20: 4878-4883.

8. Beale, E.G., J.L. Hartley and D.K. Granner. 1982. N^6,O^2-Dibutyryl cyclic AMP and glucose regulate the amount of messenger RNA coding for hepatic phosphoenolpyruvate carboxykinase (GTP). J. Biol. Chem. 257: 2022-2028.

9. Benarcerraf, B.G., G.J. Thorbecke and D. Jacoby. 1959. Effect of zymosan on endotoxin toxicity of mice. Proc. Soc. Exp. Biol. Med. 100: 796-799.

10. Berry, L.J., D.S. Smythe and L.G. Young. 1959. Effects of bacterial metabolism. I. Carbohydrate depletion and the protective role of cortisone. J. Exp. Med. 110: 389-405.

11. Berry, L.J. and D.S. Smythe. 1961. Effects of bacterial endotoxins on metabolism. III. Nitrogen excretion after ACTH as an assay for endotoxin. J. Exp. Med. 113: 83-94.

12. Berry, L.J. and D.S. Smythe. 1963. Effects of endotoxins on metabolism. VI. The role of tryptophan pyrrolase in response of mice to endotoxin. J. Exp. Med. 118: 587-603.

13. Berry, L.J. 1964. Endotoxin lethality and tryptophan pyrrolase induction in cold exposed mice. Am. J. Physiol. 207: 1058-1062.

14. Berry, L.J. and D.S. Smythe. 1964. Effects of bacterial endotoxins on metabolism. VII. Enzyme induction and cortisone protection. J. Exp. Med. 120: 721-732.

15. Berry, L.J. 1966. Effects of environmental temperature on lethality of endotoxin and its effect on body temperature in mice. Fed. Proc. 25: 1264-1270.

16. Berry, L.J., D.S. Smythe and L.S. Colwell. 1968. Inhibition of hepatic enzyme induction as a sensitive assay for endotoxin. J. Bacteriol. 96: 1191-1199.

17. Berry, L.J. 1971. Metabolic effects of bacterial endotoxins. In Microbial Toxins (S. Kadis, G. Weirbaum and S.J. Ajl, eds.), Academic Press, New York, pp. 165-208.

18. Berry, L.J. and D.F. Rippe. 1973. Effect of endotoxin on induced liver enzymes. J. Inf. Dis. 128: S118-S121.

19. Berry, L.J., R.N. Moore, K.J. Goodrum and R.E. Couch. 1979. Cellular requirements for enzyme inhibition by endotoxin in mice. In Microbiology (D. Schlessinger, ed.), American Society for Microbiology, Washington, D.C., pp. 321-325.

20. Berry, L.J. and J.E. Gaska. 1983. The role of humoral factors in endotoxin-induced reactions. In Beneficial Effects of Endotoxins (A. Nowotny, ed.), Plenum Press, New York, pp. 355-380.

21. Berry, L.J. and W.B. Huff. 1984. Antiglucorticoid effects of endotoxin. Circ. Shock. 14: 137-145.

22. Berry, L.J. and G.M. Shackleford. 1984. The molecular basis of the antagonism between bacterial endotoxins and glucocorticoids. In Adrenal Steroid Antagonism (M.K. Agarwal ed.) Walter de Gruyter & Co., New York, pp. 103-129.

23. Bértók, L. 1983. Stimulation of nonspecific resistance by radiationdatoxified endotoxin. In Beneficial Effects of Endotoxins (A. Nowotny, ed.), Plenum Press, New York, pp. 213-226.

24. Birnberg, N.C., J.C. Lissitzky, M. Hinnman and E. Herbert. 1983. Glucocorticoids regulate proopiomelanocortin gene expression in vivo at the levels of transcription and secretion. Proc. Natl. Acad. Sci. USA 80: 6982-6986.

25. Bitensky, M.W., R.E. Gorman and L. Thomas. 1971. Selective stimulation of epinephrine-responsive adenyl cyclase in mice by endotoxin. Proc. Soc. Exp. Biol. Med. 138: 773-775.

26. Boise, E. 1897. The Differential diagnosis of shock, hemorrhage, and sepsis. Tr. Am. A. Obst. 9: 433.

27. Boorsma, D.M. and J.G. Streefkerk. 1979. Periodate or glutaraldehyde for preparing peroxidase conjugates. J. Immuno. Method. 30: 245-255.

28. Bottger, I., H. Kriegal and O. Wieland. 1970. Fluctuation of hepatic enzymes important in glucose metabolism in relation to thyroid function. Eur. J. Biochem. 13: 253-257.

29. Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analy. Biochem. 72: 248-254.

30. Brake, C.M., T.E. Emerson, D.D. Holmes, M.M. Jordan, and F.D. Masucci. 1964. Role of sympatho-adrenal system in endotoxin shock. Fed. Proc. 23: 416.

31. Brake, C.M., T.E. Emerson, L.E. Wittmers and L.B. Hinshaw. 1964. Alteration of vascular response to endotoxin by adrenergic blockade. Am. J. Physiol. 207: 149-151.

32. Braude, A.I. and J.A. Rock. 1959. The syndrome of acute disseminated moniliases in adults. Arch. Int. Med. 104: 107-116.

33. Brooke, M., E.H. Kass and O. Hechter. 1961. Anti-endotoxin activity of corticosteroids. Endocrinology 69: 867-870.

34. Burnette, W.N. 1981. 'Western blotting': Electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Analy. Biochem. 112: 195-203.

35. Cannon, W.B. 1914. The emergency function of the adrenal medulla in pain and the major emotions. Am. J. Physiol. 33: 356-372.

36. Chang, H.C. and M.D. Lane. 1966. The enzymatic carboxylation of phosphoenolpyruvate. II. Purification and properties of liver mitochondrial phosphoenolpyruvate carboxykinase. J. Biol. Chem. 241: 2413-2420.

37. Chedid, L.M., M. parant, F. Boyer and R.C. Skarnes. 1964. Nonspecific host responses in tolerance to the lethal effect of endotoxins. In Bacterial Endotoxins (M. Landy and W. Braun, eds.), Rutgers Univ. Press, New York, New Jersey, pp. 500-524.

38. Chrapkiewicz, N.B., E.G. Beale and D.K. Granner. 1982. Induction of the messenger ribonucleic acid coding for phosphoenolpyruvate carboxykinase in H4-II-E cells. J. Biol. Chem. 257: 14428-14432.

39. Cimbala, M.A., W.H. Lamers, K. Nelson, J.E. Monahan, H.Y. Warran and R.W. Hanson. 1982. Rapid changes in the concentration of phosphoenolpyruvate carboxykinase mRNA in rat liver and kidney. J. Biol. Chem. 257: 7629-7636.

40. Clausen, J. 1972. Immunodiffusion in gels. In Laboratory Techniques in Biochemistry and Molecular Biology (T.S. Work and E. Work, eds.) North-Holland Publishing Co., London, pp. 427-430.

41. Colombo, G., G.M. Carlson and H.A. Hardy. 1978. Phosphoenolpyruvate carboxykinase (guanosine triphosphate) from rat liver cytosol. Separation of homogenous forms of the enzyme with high and low activity by chromatography on agarose-hexane-guanosine triphosphate. Biochem. 17: 5321-5329.

42. Condie, R.M., E.V. Staab and R.A. Good. 1962. Studies on the biologic relationship of endotoxin and other toxin proteins. I. Comparison of the properties of snake venom and endotoxin. J. Exp. Med. 115: 563-577.

43. Couch, R.E., R.N. Moore and L.J. Berry. 1979. Sensitization of tolerant mice to cold with a serum factor induced by endotoxin. J. Appl. Physiol. 46: 14-18.

44. Crafton, C.G. and N.R. DiLuzio, 1969. Relationship of reticuloendothelial functional activity to endotoxin lethality. Am. J. Physiol. 217: 736-742.

45. Crowell, J.W. and W.L. Read. 1955. In vivo coagulation - a probable cause of irreversible shock. Am. J. Physiol. 183: 565-569.

46. Crowell, J.W. and B. Houston. 1961. Effect of acidity on blood coagulation. Am. J. Physiol. 201: 379-382.

47. Dimitriu, A. 1975. Suppression of macrophage arming by corticosteroids. Cell. Immuno. 211: 79-87.

48. Dirksen, R., G.J. Wood and C.M.M. Nijhuis. 1981. Mechanism of naloxone therapy in the treatment of shock: a hypothesis. Lancet 8220: 607-608.

49. Duffy, B.J. and H.R. Morgan. 1951. ACTH and cortisone aggravation on suppression of the febrile response of rabbits to bacterial endotoxin. Proc. Soc. Exp. Biol. Med. 78: 687-689.

50. Exton, J.H. 1979. regulation of gluconeogenesis by glucocorticoids. In Glucocorticoid Hormone Action (J.D. Baxter and G.G. Rousseau, eds.), Springer Verlag, New York, pp. 535-546.

51. Euler, U.S. 1956. Suprarenal medullary secretion. In Noradrenaline (R.F. Pitts, ed.), Charles C. Thomas, Springfield, Il., pp. 259-284.

52. Faden, A.I. and J.W. Holaday. 1980. Experimental endotoxin shock: The pathophysiologic function of endorphins and treatment with opiate antagonists. J. Inf. Dis. 142: 229-238.

53. Faden, A.I. and J.W. Holaday. 1980. Naloxone treatment of endotoxin shock: Stereospecificity of physiologic and pharmacologic effects in the rat. J. Pharm. Exp. Ther. 212: 441-447.

54. Fasel, N., K. Pearson, E. Buetti and H. Diggelmann. 1982. The region of mouse mammary tumor virus DNA containing the long terminal repeat includes a long coding sequence and signals for hormonally regulated transcription. E.M.B.O. J. 1: 3-7.

55. Fearon, D.T., S. Ruddy, P.H. Schur and W.R. McCabe. 1975. Activation of the properdin pathway of complement in patients with gram-negative bacteremia. N. Engl. J. Med. 292: 937-940.

56. Felty, A.R. and C.S. Keefer. 1924. Bacillus coli sepsis: clinical study of 28 cases of blood stream infection by colon bacillus. J.A.M.A. 82: 1430-1432.

57. Fettman M.J., M.S. Hand, L.G. Chandrasena, J.L. Cleek, R.A. Mason, P.A. Brooks and R.A. Mason, P.A. Brooks and R.W. Phillips. 1984. Naloxone therapy in awake endotoxemic Yucatan minipigs. J. Surg. Res. 37: 208-225.

58. Filkins, J.P. and R.P. Cornell. 1974 Depression of hepatic gluconeogenesis and the hypoglycemia of endotoxin shock. Am. J. Physiol. 227: 778-781.

59. Foster, D.O., P.D. Ray and H.A. Lardy. 1966. Studies on the mechanism underlying adaptive changes in rat liver phosphoenolpyruvate carboxykinase. Biochem. 5: 555-562.

60. From, A.H.L., H. Gewurz, R.P. Gruninger, R.J. Pickering and W.W. Spink. 1970. Complement in endotoxin shock: effect of complement depletion on the early hypotensive phase. Infect. Immun. 2: 38-41.

61. Geller, P., E.R. Merrill and E. Jawetz. 1954. Effects of cortisone and antibiotics on lethal action of endotoxins in mice. Proc. Soc. Exp. Biol. Med. 86: 716-719.

62. Gilbert, R.P. 1960. Mechanism of the hemodynamic effects of endotoxin. Physiol. Rev. 40: 245-279.

63. Gilbert, V.E. and A.J. Braude. 1962. Reduction of serum complement in rabbits after injection of endotoxin. J. Exp. Med. 116: 477-490.

64. Goldstein, A. and P.J. Lowery. 1975. Effect of opiate antagonist naloxone on the body temperature in rats. Life Sciences 17: 927-932.

65. Goodrum, K.J. and L.J. Berry. 1978. The effect of GAF on hepatoma cells. Proc. Soc. Exp. Biol. Med. 159: 353-363.

66. Goodrum, K.J. and L.J. Berry. 1979. The use of Reuber hepatoma cells for the study of lipopolysaccharide - induced macrophage factor. Lab. Invest. 41: 174-180.

67. Gunn, J.M., S.M. Tilghman, R.W. Hanson, L. Reshef and F.J. Ballard. 1975. Effects of cyclic adenosine monophosphate, dexamethasone and insulin on phosphoenolpyruvate carboxykinase synthesis in Reuber H-35 hepatoma cells. Biochem. 14: 2350-2357.

68. Gunn, W.C. 1914. The variation in the amount of complement in the blood in some acute infectious diseases and its relation to the clinical features. J. Pathol. Bacteriol. 19: 155-181.

69. Guillemin, R., T. Vargo, J. Rossier, S. Minick, N. Ling, C. Rivier, W. Vale and F. Bloom. 1979. B-Endorphin and adrenocorticotropin are secreted concomitantly by the pituitary gland. Science 197: 1367-1369.

70. Hall, W.H. and D. Gold. 1955. Shock associated with bacteremia. Arch. Int. Med. 96: 403-412.

71. Halpern, B.N., B. Benacerraf and M. Briot. 1952. Potentiation by adrenaline of protective effect of cortisone on histamine toxicity in adrenalectomized mice. Proc. Soc. Exp. Biol. Med. 79: 37-39.

72. Hanson, R.W. and A.J. Garber. 1972. Phosphoenolpyruvate carboxykinase. I. Its role in gluconeogenesis. Am. J. Clin. Nutr. 25: 1010-1021.

73. Harel, M. and S. Amir. 1983. Role of endorphins in endotoxin - induced hyperglycemia in mice. Neuropharm. 22: 1117-1119.

74. Heroux, O. and J.S. Hart. 1954. Adrenal cortical hormone requirement of warm and cold acclimated rats after adrenalectomy. Am. J. Physiol. 178: 449-452.

75. Higgins, S.J. and U. Gehring. 1978. Molecular mechanisms of steroid hormone action. Adv. Cancer Res. 28: 313-397.

76. Hinshaw, L.B., M.M. Jordan and J.A. Vick. 1961. Mechanism of histamine release in endotoxin shock. Am. J. Physiol. 200: 987-989.

77. Hinshaw, L.B., M.M. Jordan and J.A. Vick. 1961. Histamine release and endotoxin shock in the primate. J. Clin. Invest. 40: 1631-1637.

78. Hinshaw, L.B., T.E. Emerson, P.F. Iampietro and C.M. Brake. 1962. A Comparative study of the hemodynamic actions of histmine and endotoxin. Am. J. Physiol. 203: 600-606.

79. Hinshaw, L.B., J.A. Vick, M.M. Jordan and L.E. Wittmers. 1962. Vascular changes associated with development of irreversible endotoxin shock. Am. J. Physiol. 202: 103-110.

80. Hinshaw, L.B., C.M. Brake, T.E. Emerson, M.M. Jordan and F.D. Masucci. 1964. Participation of sympathoadrenal system in endotoxin shock. Am. J. Physiol. 207: 925-930.

81. Holaday, J.W. and A.I. Faden. 1978. Naloxone reversal of endotoxin hypotension suggests role of endorphins in shock. Nature 275: 450-451.

82. Holaday, J.W., E. Wei, H.H. Loh and C.H. Li. 1978. Endorphins may function in heat adaption. Proc. Natl. Aca. Sci. USA 75: 2923-2927.

83. Holday, J.W. 1983. Endorphins in shock and spinal injury; therapeutic effects of naloxone and thyrotropin-releasing hormone. In Progress in Clinical and Biological Research (A.M. Lefer and W. Schumer, eds), Alan R. Liss Inc., New York, pp. 167-184.

84. Iynedjian, P.B. and R.W. Hanson. 1977. Increase in the level of functional messenger RNA coding for phosphoenol-pyruvate carboxykinase (GTP) during induction of cyclic adenosine 3':5'-monophosphate. J. Biol. Chem. 252: 655-662.

85. Iynedjian, P.B. and R.W. Hanson. 1977. Messenger RNA for renal phosphoenolpyruvate carboxykinase (GTP) J. Biol. Chem. 252: 8398-8403.

86. Iynedjian, P.B. 1979. Purification of phosphoenolpyru-vate carboxykinase (GTP) by affinity chromatography on aga-rose-hydrazide GTP. Enzyme 24: 366-373.

87. Jackson, H. and S. Oleesky. 1946. An unusual case of Weil's disease. Br. J. Med. 2: 813-814.

88. Jacob, H.S. 1980. Complement-induced vascular leukocy-tosis. Arch. Path. Lab. Med. 104: 617-620.

89. Jacobson, E.D., B. Mehlman and J.P. Kalas. 1964. Vaso-active mediators as the 'trigger mechanism' of endotoxin shock. J. Clin. Invest. 43: 1000-1013.

90. Kadowitz, P.J. and A.C. Yard. 1970. Circulatory effects of hydrocortisone and protection against endotoxin shock in cats. Eur. J. Pharmacol. 9: 311-318.

91. Kaplowitz, L.G., J.V. Lange, J.J. Fischer and D.H. Walker. 1983. Correlation of Rickettsial titers, circula-tory endotoxin, and clinical features in Rocky Mountain spot-ted fever. Arch. Int. Med. 143: 1149-1151.

92. Krone, W., W.B. Huttner, H.J. Seitz, and W. Tarnowski. 1976. Effect of cold exposure on phosphoenolpyruvate carboxykinase (GTP) activity and cyclic AMP concentrations in livers of starved rats. Biochem. et Biophys. Acta. 444: 694-703.

93. Kuida, H., R.P. Gilbert, L.B. Hinshaw, J.G. Brunson and M.B. Visscher. 1961. Species differences in effect of gram-negative endotoxin in circulation. Am. J. Physiol. 200: 1197-1202.

94. Kun, E. 1948. Effect of bacterial endotoxins on glycogen synthesis. Proc. Soc. Exp. Biol. Med. 68:: 496-500.

95. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

96. Landy, M. and W.P. Weidanz. 1964. Natural antibodies against gram-negative bacteria. In Bacterial Endotoxins (M. Landy and W. Braun, eds.). Quinn and Boden Co., Inc., New Jersey, pp. 275-290.

97. Lefer, A.M. 1983. Pharmacologic and surgical modulation of myocardial depressant factor formation and action during shock. In Molecular and Cellular Aspects of Shock and Trauma (A.M. Lefer and W. Schumer, eds.), Alan R. Liss, Inc., New York, pp. 111-123.

98. Lemaire, I., R. Tseng and S. Lemaire. 1978. Systemic adminstration of B-endorphin: potent hypotensive effect involving a serotonergic pathway. Proc. Natl. Acad. Sci. USA 75: 6240-6242.

99. Levitin, H., M.I. Kendrick and E.H. Kass. 1956. Effect of route of administration on protective action of corticosterone and cortisol against endotoxin. Proc. Soc. Exp. Biol. Med. 93: 306-309.

100. Long, C.N.H., B. Katzin and E.G. Fry. 1940. The adrenal cortex and carbohydrate metabolism. Endocrinology 26: 309-344.

101. McCallum, R.E. and L.J. Berry. 1972. Mouse liver fructose-1,6-diphosphatase and glucose-6-phoshatase activities after endotoxin poisoning. Infect. and Immun. 6: 883-885.

102. McCallum, R.E. and L.J. Berry. 1973. Effects of endotoxin on gluconeogenesis, glycogen synthesis and liver glycogen synthase. Infect. Immun. 7: 642-654.

103. Maclean, L.D., M.H. Weil, W.W. Spink and M.B. Visscher. 1956. Canine intestinal and liver weight changes induced by E. Coli. endotoxin. Proc. Soc. Exp. Biol. Med. 92: 602-605.

104. Mains, R.E., B.A. Eipper and N. Ling. 1977. Common precursor to corticotropins and endorphins. Proc. Natl. Acad. Sci. USA 74: 3014-3018.

105. Martin, W.J. and M.C. McKenry. 1962. Fifty-nine cases of bacteremic shock due to gram-negative enteric bacilli. Med. Clin. North Amer. 46: 1073-1097.

106. Masucci, F.D., and L.B. Hinshaw. 1964. Evaluation of protection with phenoxybenzamine against lethal endotoxin shock. Proc. Soc. Exp. Biol. Med. 116: 1057-1060.

107. Mencher, D. and L. Reshef. 1979. Effect of triamcinolone on renal and hepatic phosphoenolpyruvate carboxykinase in the newborn rat. Eur. J. Biochem. 94: 581-589.

108. Menten, M.L. and H.M. Manning. 1924. Blood sugar studies on rabbits infected with organisms of the enteritis-paratyphoid group. J. Med. Res. 44: 675-676.

109. Mills, L.C. 1971. Corticosteroids in endotoxic shock. Proc. Soc. Exp. Biol. Med. 138: 507-511.

110. Moberg, G.P. 1971. Site of action of endotoxin on hypothalamic-pituitary-adrenal axis. Am. J. Physiol. 220: 397-400.

111. Moore, R.N, K.J. Goodrum and L.J. Berry. 1976. Mediation of endotoxic effects of macrophages. RES J. Reticuloendothel. Soc. 19: 187-197.

112. Moore, R.N. K.J. Goodrum, L.J. Berry and J.R. McGhee. 1977. An abnormal response of nude mice to endotoxin. RES J. Reticuloendothel. Soc. 21: 271-277.

113. Moore, R.N. B.A. Johnson and L.J. Berry. 1977. Nutritional effects of salmonellosis in mice. Amer. J. Clin. nutr. 30: 1289-1293.

114. Moore, R.N. K.J. Goodrum, R.E. Couch and L.J. Berry. 1978. Elicitation of endotoxin effects on C3H/HeJ mice with GAF and partial characterization of the factor. Infect. Immun. 19: 79-86.

115. Moore, R.N., K.J. Goodrum, R.E. Couch and L.J. Berry. 1978. Factors affecting macrophage function: GAF. RES J. Reticuloendothel. Soc. 23: 321-332.

116. Nagai, K. and H. Nakagawa. 1972. Effect of thyroxine on phosphoenolpyruvate carboxykinase in rat liver in normal and cold environment. J. Biochem. 71: 125-131.

117. Nacheles, H., P. Dommers, M. Weiner, W.H. Olson and W. Rychel. 1942. Depression of gastric motility without elevation of body temperature following the injections of pyrogens. Am J. Physiol. 137: 22-29.

118. Nordlie, R.C., F.E. Varricchio and D.D. Holton. 1965. Effects of altered hormonal states and fasting on rat liver mitochondrial phosphoenolpyruvate carboxykinase levels. Biochem. et Biophys. Acta. 97: 214-221.

119. Nowotny, A. 1979. Isolation of IgG fraction. In Basic Exercises in Immunochemistry (A. Nowotny, ed.), Springer-Verlog, New York, pp. 8-10.

120. Nowotny, A. 1979. Immunoelectrophoresis. In Basic Exercises in Immunochemistry. (A. Nowotny, ed.), Springer-Verlog, New York, pp. 235-237.

121. Noyes, H.E., C.R. McInturf and G.J. Blahuta. 1959. Studies on distribution of Escherichia coli endotoxin in mice. Proc. Soc. Exp. Biol. Med. 100: 65-68.

122. Parker, M.M. and J.E. Parrillo. 1983. Septic shock, hemodynamics and pathogenesis. J.A.M.A. 250: 3324-3327.

123. Phillips, L.J. and L.J. Berry. 1970. Hormonal control of mouse liver phosphoenolpyruvate carboxykinase rhythm. Am. J. Physiol. 219: 697-701.

124. Previte, J.J. and L.J. Berry. 1962. The effect of environmental temperature on the host-parasite relationship in mice. J. Inf. Dis. 110: 201-209.

125. Previte, J.J. and L.J. Berry. 1963. Studies on the potentiation of endotoxin in mice by exposure to cold. J. Inf. Dis. 113: 43-51.

126. Reshef, L., R.W. Hanson and F.J. Ballard. 1970. A possible physiological role for glyceroneogenesis in rat adipose tissue. J. Biol. Chem. 245: 5979-5984.

127. Reshef, L. and R.W. Hanson. 1972. The interaction of catecholamines and adrenal corticosteroids in the induction of phosphopyruvate carboxylase in rat liver and adipose tissue. Biochem. J. 127: 809-818.

128. Rifkind, D. 1976. Prevention by polymyxin B of endotoxin lethality in mice. J. Bact. 93: 1463-1464.

129. Ringfold, G.M., K.R. Yamamoto, J.M. Bishop and H.E. Varmus. 1977. Glucocorticoid-stimulated accumulation of mouse mammary tumor virus RNA: Increased rate of synthesis of viral RNA. Proc. Natl. Acad. Sci. USA 74: 2879-2883.

130. Rippe, D.F. and L.J. Berry. 1972. Effect of endotoxin on the activation of PEPCK by tryptophan. Infec. Immun. 6: 97-98.

131. Rippe, D.F. and L.J. Berry. 1972. Study of inhibition of FEPCK by endotoxin with radial immunodiffusion. Infect. Immun. 6: 766-772.

132. Rippe, D.F. and L.J. Berry. 1973. Immunological quantitation of hepatic tryptophan oxygenase in endotoxin poisoned mice. Infect. Immun. 8: 534-539.

133. Rippe, D.F. and Berry, L.J. 1973. Metabolic manifestations of lead acetate sensitization to endotoxin in mice. RES J. reticuloendothel. Soc. 13: 527-535.

134. Rognstad, R. 1979. Rate-limiting steps in metabolic pathways. J. Biol. Chem. 254: 1875-1878.

135. Rosenberg, J.C., R.C. Lillehei, W.H. Moran and B. Zimmermann. 1959. Effect of endotoxin on plasma catechol amines and serotonin. Proc. Soc. Exp. Biol. Med. 102: 335-337.

136. Rosenthal, S.M. 1943. Experimental chemotherapy of burns and shock. IV. Production of traumatic shock in mice. V. Therapy with mouse serum and sodium salts. Public Health Reports. 58: 1429-1436.

137. Rossier, J., E.D. French, C. Rivier, N. Ling, R. Guillemin and F.E. Bloom. 1978. Foot-shocked induced stress increases B-endorphin levels in blood but not brain. Nature 270:618-620.

138. Rousseau, G.G. 1984. Control of gene expression by glucocorticoid hormones. Biochem. J. 224: 1-12.

139. Sanford, J.P., J.A. Barnett and C. Gott. 1960. A mechanism of the glycogenolytic action of bacterial endotoxin. J. Exp. Med. 112: 97-105.

140. Sayers, G. 1950. Adrenal cortex and homeostasis. Physiol. Rev. 30: 241-320.

141. Schayer, R.W. 1960. Relationship of induced histidine decarboxylase activity and histamine synthesis to shock from stress and from endotoxin. Am. J. Physiol. 198: 1187-1192.

142. Schayer, R.W. 1962. Role of induced histamine in tourniquet shock in mice. Am J. Physiol. 203: 412-416.

143. Schirger, A., W.J. Martin and D.R. Nichols. 1956. Streptococcal bacteremia without endocarditis: clinical observations. Ann. Int. Med. 45: 1001-1009.

144. Schirger, A., W.J. Martin and D.R. Nichols. 1957. Micrococcal bacteremia without endocarditis: clinical data and therapeutic considerations in 109 cases. Ann. Int. Med. 47: 39-48.

145. Schrauwen, E.,G. Vanderplassche and A. Kouvenaghel. 1984. Effects of high dose opiate antagonists in porcine endotoxic shock. IRCS, Med. Sci. 12: 144-145.

146. Seubert, W., H.U. Kenning, W. Schomer and M. L'Age. 1968. Effects of cortisol on the levels of metabolites and enzymes controlling glucose production from pyruvate. In Advances in Enzyme Regulation (G. Weber, ed.), Pergamon Press, New York, pp. 153-180.

147. Shands, J.W., V. Miller, H. Martin and V. Senterfitt. 1969. Hypoglycemic activity of endotoxin. II. Mechanisms of the phenomenon in BCG-infected mice. J. Bact 98: 494-501.

148. Shrago, E., H.A. Lardy, R.C. Nordlie and D.O. Foster. 1963. Metabolic and hormonal control of phosphoenolpyruvate carboxykinase and malic enzyme in rat liver. J. Biol. Chem. 238: 3188-3192.

149. Shrago, E., J.W. Young and H.A. Lardy. 1967. Carbohydrate supply as a regulator of rat liver phosphoenolpyruvate carboxykinase activity. Science: 158: 1572-1573.

150. Smith, D.J. 1951. In vitro action of cortisone on vasoconstriction due to histamine. Proc. Soc. Exp. Biol. Med. 77: 534-536.

151. Spink, W.W. and J. Vick. 1961. A labile serum factor in experimental endotoxin shock: cross-transfusion studies in dogs. J. Exp. Med. 114: 501-508.

152. Spink, W.W., R.B. Davis, R. Potter and S. Chartrand. 1964. The initial stage of canine endotoxin shock as an expression of anaphylactic shock: studies on complement titers and plasma histamine concentrations. J. Clin. Invest. 43: 696-704.

153. Thomas, L. 1954. The physiological disturbances produced by endotoxins. Ann. Rev. Physiol. 16: 467-490.

154. Tilghman, S.M., J.M. Gunn, L.M. Fisher and R.W. Hanson. 1975. Deinduction of phosphoenolpyruvate carboxykinase (guanosine triphosphate) synthesis in Reuber H35 cells. J. Biol. Chem. 250: 3322-3329.

155. Tilghman, S.M., R.W. Hanson and F.J. Ballard. 1976. In Gluconeogenesis: Its Regulation in Mammaliam Species. (R.W. Hanson and M.A. Mehlman, eds.), John Wiley and Sons, New York, pp. 47-91.

156. Till, G.O., K.J. Johnson, R. Kunkel and P.A. Ward. 1982. Intravascular activation of complement and acute lung injury. J. Clin. Invest. 69: 1126-1135.

157. Towbin, H., T. Staehelin and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. USA 76: 4350-4354.

158. Tsang, V.C.W., B.C. Wilson and J.M. Peralta. 1983. Quantitative, single-tube, kinetic-dependent enzyme -linked immunosorbant assay (k-ELISA). Meth. Enz. 92: 391-403.

159. Turner, M.M. and L.J. Berry. 1963. Inhibition of gastric emptying in mice by bacterial endotoxins. Am. J. Physiol. 205: 1113-1116.

160. Ucker, D.S., G.L. Firestone and K.R. Yamamoto. 1983. Glucocorticoids and chromosomal position modulate murine mammory tumor virus transcription by effecting efficiency of promoter utilization. Mol. Cell. Biol. 3: 551-561.

161. Ulevitch, R.J., C.G. Cochrane, P.M. Henson, D.C. Morrison and W.F. Doe. 1975. Mediation systems in bacterial lipopolysaccharide-induced hypotension and disseminated intravascular coagulation. I. The role of complement. J. Exp. Med. 142: 1570-1590.

162. Utter, M.F. and K. Kurahashi. 1954. Purification of oxaloacetic carboxylase from chicken liver. J. Biol. Chem. 207: 787-802.

163. Waisbren, B.A. 1951. Bacteremia due to gram-negative bacilli other than Salmonella. Arch. Int. Med. 88: 467-488.

164. Warren, H.Y., M.A. Cimbala, K. Felz, J.E. Monahan, J.P. Lewis and R.W. Hanson. 1981. Identification of DNA clone to phosphoenolpyruvate carboxykinase (GTP) from rat cytosol. J. Biol. Chem. 256: 10224-10227.

165. Weber, K. and M. Osborn. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. 244: 4406-4412.

166. Weil, M.H., L.D. MacLean, M.B. Visscher and W.W. Spink. 1956. Studies on the circulatory changes in the dog produced by endotoxin from gram-negative microorganisms. J. Clin. Invest. 35: 1191-1198.

167. Weil, M.H. and W.W. Spink. 1957. A comparison of shock due to endotoxin with anaphylactic shock. J. Lab. Clin. Med. 50: 501-515.

168. Weil, M.H., H. Schubin and M. Biddle. 1964. Shock caused by gram-negative microorganisms. Ann. Int. Med. 60: 384-400.

169. Wicks, W.D. 1969. Induction of heptic enzymes by adenosine 3' ,5'-monophosphate in organ culture. J. Biol. Chem. 244: 3941-3950.

170. Wicks, W.D., C.A. Barrett and J.B. Mckibbin. 1974. Interaction between hormones and cyclic AMP in regulating specific hepatic enzyme synthesis. Fed. Proc. 33: 1105-1111.

171. Wolfe, R.R., D. Elahi and J.J. Spitzer. 1977. Glucose kinetics in dogs following a lethal dose of endotoxin. Metabolism 26: 847-850.

172. Wolff, S.M. and J.V. Bennett. 1974. Gram negative rod bacteremia. N Engl. J. Med. 291: 733-734.

173. Yelich, M.R. and J.P. Filkins. 1982. Insulin hypersecretion and potentiation of endotoxin shock in the rat. Circ. Shock. 9: 589-603.